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Deoxyribonucleic Acid (DNA) Autodegradation from Surface Weathered Bone as an Indicator of Postmortem Interval

by

Dean Derek Bamber ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of *Masters of Arts*

Department of Anthropology

Edmonton, Alberta

Fall 1995

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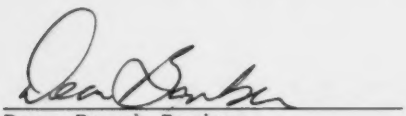

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The wilderness had found them out early,
and taken on him a terrible vengeance...
it
had whispered to him things about himself
which he did not know, things of which he
had no conception till he took counsel
with great solitude...
and the whisper had proved irresistibly
fascinating. It echoed loudly within him
because he was hollow at the core...

Joseph Conrad
Heart of Darkness

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *"Deoxyribonucleic Acid (DNA) Autodegradation from Surface Weathered Bone as an Indicator of Postmortem Interval"* submitted by Dean Derek Bamber in partial fulfilment of the requirements for the degree of *Masters of Arts*.

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ABSTRACT

This study examines the feasibility of using the quantity and quality of DNA extracted from surface weathered compact bone as a measure of the time elapsed since death.

Seven samples of *Bos taurus* compact bone were collected from adult females that had died between 14 days and 18 years earlier. Each sample was processed and the DNA was then extracted in such a way as to maximize total DNA yield and avoid the loss of low molecular weight fragments. The samples were run on a 2% agrose gel stained with ethidium bromide. A digitalized density scan was obtained for each sample and was used to quantify and qualify the DNA extracted.

Results showed that as the postmortem interval increased the ability to collect high molecular weight DNA of any quantity rapidly decreased over the first 22 months, after that time only a very small quantity of high molecular weight DNA, suspected to be bacterial and/or algal contamination, could be collected. A relatively constant decrease in the average DNA fragment size can be observed up to approximately this 22 month period at which point the relative proportion of bovine genomic DNA to the microbial DNA contamination makes it impossible to characterize further DNA degradation.

Although the presence of this microbial DNA made it difficult to conclude definitively that the quantity and quality of DNA can be used to estimate the time elapsed since death, this research has shown that predictable trends do exist in the DNA degradation process. With only slight modifications to experimental design, a workable model for the use of DNA condition as a measure of the postmortem interval is likely.

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LIST OF SYMBOLS, NOMENCLATURE AND ABBREVIATIONS

bp:	base pairs
C:	Celsius
cm:	centimetre
Dly:	daily
DNA:	deoxyribonucleic acid
deg:	degrees
fem:	female
G:	gravities
g:	gram
hrly:	hourly
humid:	humidity
log:	logarithm
Mx:	maximum
med:	medial
microgr:	microgram
mm:	millimetres
min:	minutes
Mn:	minimum
N:	not available
NIH:	National Institutes of Health
PM:	postmortem
Prcp:	precipitation
prox:	proximal
RFLP:	Restriction Fragment Length Polymorphism
Rn:	rain
RNA:	ribonucleic acid
Snw:	snow
TM:	Trade Mark
Temp:	temperature
Tot:	total

CHAPTER ONE

INTRODUCTION

During the later stages of body decomposition, identification of human remains can be extraordinarily difficult. For bodies stripped of distinguishable artifacts, it is frequently by mere chance that remains are related to missing individuals and then identified through circumstantial evidence (Reichs, 1986: XXV). Determination of time elapsed since death, in these situations, is generally the most important step in the identification process.

The decomposition rates of human remains vary with environment and antemortem condition of the individual (Haglund, 1993). This wide variation is demonstrated by the fact that in some situations, bodies have become completely skeletonized in as little as two weeks, whereas in others, they can exist in a state of excellent preservation for hundreds, even thousands of years (Stewart, 1979). As of yet, there are no well accepted rules for determining the postmortem interval by decomposition rates alone. The multiple variables involved so far have made quantifiable determinations impossible. In the majority of forensic cases, experienced investigators are forced to simply guess, producing only rough estimations of the time since death, with an increasing margin of error as the decomposition process advances (Reichs, 1986: XXV).

Efforts to rectify this shortcoming in the Forensic Sciences have produced some reasonable, but relatively inaccurate, investigative methods. Rodriguez (1983,1985), Duffy (1991) and Vass (1992) have made considerable advances in illuminating the process of soft tissue

decomposition. Goff (1988), and Greenberg (1990,1991) have correlated stages of insect infestation on decomposing bodies with time since death; and Lundquist (1963), Villanueva (1976), Castellano et al. (1984) and Yoshino et al. (1991) have defined postmortem changes in human compact bone through a variety of techniques, and correlated them to the time elapsed since death. The results of these varied approaches, however, are strongly influenced by environmental and antemortem conditions, and therefore, still only produce rough estimations of the postmortem period.

Numerous other techniques, from histological examination of decomposing tissue (Janssen, 1977; pages 13-53) to biochemical analyses (Querido 1988,1990 a&b, 1991; Stephens et al., 1987; Sparks et al., 1989; Nowak et al., 1989; Endo et al., 1990; Nagata et al., 1990; Rognum et al., 1991 and Gamero-Lucas et al., 1992) and study of the decay of associated materials (Morse et al., 1983), have been tried as measures of the postmortem interval. All have been shown to depend on the degree of body decomposition (which fluctuates with the environment), and therefore, are under the influence of non quantifiable factors. Currently, there are no methods available for determining time elapsed since death that are relatively independent of environmental fluctuations and that express a relatively constant change over time. Some recent research, however, is beginning to suggest new possibilities.

Deoxyribonucleic acid (DNA), is found in every nucleated cell in the human body. It carries the genetic code which directs and controls the synthesis of all body proteins. The ability of a cell to survive and divide depends on the integrity of its DNA. Permanent DNA damage may lead to cell death. DNA may be damaged by ionizing radiation, harsh chemicals and enzymes but is relatively resistant to mechanical

breakage. (Suzuki et al., 1986: 185).

When a person dies, cell autolysis releases many different enzymes into the cytoplasm inevitably include ones specialized for cutting DNA. This results in a relatively rapid fractionation of the molecular chain. However, as the postmortem interval increases these nucleases lose activity and the disintegration process slows and eventually comes to a stop. In the majority of cases, any further disintegration is dependent on mechanical breakage or ionizing radiation degradation alone. Probability suggests mechanical breakage would occur at a relatively slow and constant rate over time, decreasing as the average molecule size decreases. This process should be relatively independent of environmental variables once enzyme activity ceases, therefore, it is my hypothesis that the measurement of the average size of the DNA fragments and the quantity of DNA remaining should correlate with the time elapsed since death. Recently, research into the postmortem stability of DNA (Bar et al., 1988), and attempts to apply it to the determination of elapsed time since death within the range of weeks postmortem (Perry et al., 1988), have produced evidence supporting this theory.

This thesis directly examines the feasibility of using the postmortem quantity and condition of DNA extracted from surface weathered bone in the range of months to years postmortem as an indicator of the time elapsed since death. Using a number of new and modified methods, DNA with a postmortem age of 14 days to 18 years was extracted from *Bos taurus* compact bone and analysed to determine the quality and quantity of DNA present in each specimen. This information was then used to support or negate the hypothesis that it directly

reflects the postmortem age of the specimen.

The significance of this research cannot be fully understood without placing it in the context of the science as a whole, therefore, chapter two examines the major existing methods for estimating time elapsed since death. Chapter three details the theory and existing evidence supporting the hypothesis that is the basis for this research. Chapters four and five outline, in some detail, the development and application of the experimental design used in this study, and then examines the results obtained. Although viewed as preliminary, obvious trends are visible, and therefore, will be shown as support for the hypothesis this thesis investigates. Finally, chapter six will present the conclusions that can be drawn from this work and will cover recommendations for further research on the topic.

CHAPTER TWO

HISTORICAL REVIEW: EXISTING METHODS FOR THE ESTIMATION OF POSTMORTEM INTERVAL

Methods used to estimate time elapsed since death are invariably tied to the decomposition process and/or the condition of osseous remains. All standard methods attempt to correlate the stage of decomposition, whether characterised biochemically, by general appearance, or through the activity of insects, with the amount of time required to reach that condition.

Studies investigating the rates of decomposition on human remains began as early as the late nineteenth century with Motter's descriptions of the order of disappearance of tissues and organs (Motter, 1898). By the early twentieth century the topic became, strangely enough, very popular among German anatomists, resulting in a landslide of information of the subject and some understanding of the variability involved in the process (Lundquist, 1963; page 254). Inevitably, the method and technology involved in the studies became considerably more sophisticated, but the results and the conclusions were the same. Advances in science allowed the ability to measure uncertainty in the finest detail, but did not help in improving the accuracy in estimating time since death. As Dr. Graeme Dowling, Chief Medical Examiner for the Province of Alberta, has stated, "as the science stands today, the best we can do for the estimation of time since death is to say that a person died sometime between the time they were last seen alive and the time they were found dead" (Graeme Dowling M.D., personal communication).

BIOCHEMICAL ANALYSIS AND ESTIMATION OF TIME SINCE DEATH

All biochemical techniques for estimating the time elapsed since death are based on the ability to quantify chemical postmortem changes and relate them to time. Most research involves relating 'living' concentrations of chemicals to postmortem concentrations in various body fluids. In particular, the changes in ion concentration within the cerebrospinal fluid and vitreous humor have been studied with considerable vigour (Durlacher et al., 1953; Mason, 1951; Murray, 1958; Janssen, 1977; Querido et al., 1988, 1990 a&b, and 1991; Endo et al., 1992).

During life, cells maintain specific chemical gradients at levels required for biological activity. The nervous system, for example, relies on differences in the concentration of potassium and sodium across cell membranes to transmit neural signals. Potassium is actively pumped into the cells, while sodium is actively pumped out. After death, this energy requiring sodium and potassium pump ceases to function, due to a lack of adenosine triphosphate (ATP), the essential molecule for all energy requiring processes. After a short time, the cells autolyze as a result of lysosomal enzymes being released into to the cytoplasm and potassium accumulates in the extracellular fluids. As the postmortem interval increases more and more cells autolyse and the potassium concentration in the extracellular fluids increases proportionally. Attempts to relate the increase in potassium concentration in the vitreous humor and cerebrospinal fluid with elapsed time are demonstrated in the work of Durlacher et al. (1953), Mason (1951), Murray (1958), Janssen (1977), Querido et al. (1988, 1990 a&b, 1991) and Endo et al. (1992).

The rate of autolysis (cell autodigestion) is tissue specific and environmentally influenced. Cells containing higher concentrations of lytic enzymes will tend to autodigest first, and higher temperatures tend to optimize the activity of lytic enzymes so cells will autolyse sooner. Furthermore, tissues with pathologies can often be affected by antemortem changes resulting in variations in their rates of autolysis after death.

A number of variations on this same scheme exist, however, the underlying process is the same; chemical gradients formed during life are used to gauge cell autolysis after death. Chemical concentrations are measured and then correlated to predetermined norms for different periods in the postmortem interval, producing an estimation of the postmortem interval.

One of the earliest biochemical techniques involved the measurement of free amino-acid nitrogen concentrations in the blood and spinal fluid (Pucher and Burd, 1925). It was noted that within three to four hours after death, there was little increase in the concentrations of these chemicals, but twelve to eighteen hours after death the concentrations had more than doubled and continued to rise up to eighty hours postmortem.

Although it was recognized by the researcher's themselves that these methods could only be used for "rough estimations", other attempts at finding a quantifiable measure of time elapsed since death include the work of Jetter et al. (1943, 1949), who studied the increase in chloride concentrations in the vitreous humor; Bollinger and Carrodus (1938), and Naumann (1950), who studied increases in creatine concentrations in the cerebrospinal fluid; Praetorius et al. (1957), who

studied xanthine and hypoxanthine concentrations in the cerebrospinal fluid; and Schleyer (1957), who studied increases in ammonia concentrations in the peripheral veins. Studies were also conducted looking at inorganic phosphates, lactic acid, lipases, histidines and serum enzyme concentrations as potential indicators of time since death. The problem was that all methods measured the same thing, the rate of cell autolysis.

Since cell autolysis rates and characteristics vary under different conditions, its use to estimate time since death is usually problematic. This very credible research has produced no reliable estimators of the postmortem interval.

DECOMPOSITION AS AN INDICATOR OF TIME SINCE DEATH

Using the degree of decomposition as an estimator of the postmortem interval basically employs direct observation and considerable experience to derive an estimate of the length of time a body has been exposed to the environment. The method attempts to account for variation in climate, soil, water, insects, animals and vegetation and predict the effects of these factors on the decomposition of human remains. Close attention is given to the presence of soft tissue remaining on bones, adipocere formation, bone weathering, bleaching, cracking, mineralization, etc., and any relevant factors at the scene (Reichs, 1986; page XXV). In short, this method is dependant on the use of extensive forensic experience to estimate subjectively time of death. Conclusions are not based on quantifiable and replicable data (Rodriquez et al., 1985).

Despite all attempts to account for uncontrollable influences, this technique is also subject to considerable environmental variability. Variation in climate, like freezing and thawing or hot dry temperatures, over many seasons can make human remains look centuries old when the person may have died within months. A frozen or mummified body, on the other hand, can turn up hundreds of years after death, and look only days old. Kijima et al. (1992), for example, found that a severed head estimated to be from two weeks to six months old by experienced investigators, turned out to be the remains of a person who had died over twenty-two months earlier. The recent discovery of the "Iceman" in Northern Italy demonstrates this problem more precisely.

The "Iceman", as he has come to be known, was found by a group of hikers on a retreating glacier in the Italian Alps along the Austrian border. A forensic pathologist was called to the scene, but not appreciating the age of the body, considered the remains that of a lost hiker. As a result, the body was recovered in a manner not considered "optimal" for archaeological remains, damaging portions of the body in the process. During the excavation, however, artifacts then thought to be associated with the Bronze Age were recovered and the body was thus recognized as a significant archaeological find. Subsequent chronometric dating has determined this "lost hiker" is over 5000 years old. It is quite likely that if archaeological artifacts had not been discovered along with these remains this amazing discovery could have ended up in an Austrian morgue, never being claimed and never being recognized for what it really is. The "Iceman" by phyical appearance is better representive of a body a few years old than it is of an individual over 5000 years past his prime (Spindler, 1993).

Recently, attempts have been made to produce more objective and quantifiable results from the decomposition rates of bodies as they apply to the postmortem interval. Duffy et al. (1991), looked at putrefaction of dental pulp under different environmental conditions, and Rodriguez et al. (1985) and Vass et al. (1992), looked at using soil conditions and the degree of ion leaching from decomposing human remains as a measure of time since death. Both studies were significantly influenced by variation in the estimates of postmortem period and no consistent results were obtained. Estimating the time elapsed since death by decomposition alone is extremely subjective. It is not presently quantifiable, and therefore, cannot be subject to independent verification. In an investigative situation these weaknesses make results produced by this method very difficult to prove and at best marginally usable in a medical-legal context.

INSECT INFESTATION AS AN INDICATOR OF TIME SINCE DEATH

The estimation of time since death through correlation with the life stages, or infestation stages, of insects has been used for a number of years. The method is based on the observation that different necrophagous insects will tend to infest a decaying body during different times of the decomposition process. *Diptera calliphoridae* (or blowflies), for example, have been known to infest bodies within hours after death while *Tenebriode* beetles, typically, will not infest bodies until only dry tissue remains, often three plus years after decay begins (Smith, 1986: 16). Studying the stage of larval development and insect type on the bodies, therefore, can give a rough estimation of the period since death (Greenberg, 1990 & 1991).

Entomological methods have been shown to be good indicators of time since death for bodies in advanced states of decay, and under reasonably constant climatic conditions. However, factors, such as seasonal freezing and thawing, humidity, winds, submersion in water, burial, position of the body, or even the amount of clothing worn, substantially decrease the effectiveness of entomological estimation. The process of freezing and thawing, as well as these other variables, tend to impede insect action on bodies. For example, in Edmonton, a body deposited in November, but found the following June, using entomological data, would be estimated to be the same age as a body deposited in April and located in June. As a result, predictions of postmortem interval using insect infestation for areas such as Edmonton, or anywhere else in Canada, for that matter, cannot be used unless supported by independent evidence (Graeme Dowling M.D., Chief Medical Examiner for the Province of Alberta, personal comunication).

A major weakness in the entomological approach to postmortem interval estimation is that the stages of insect infestation on bodies are influenced by the degree of decomposition, and therefore, prone to error caused by the many other variables influencing it: antemortem condition of the individual, position of the body, presence of clothing, moisture, wind, cold, etc.

THE ANALYSIS OF BONE AS AN INDICATOR OF POSTMORTEM INTERVAL

The complete loss of soft tissues from human remains greatly reduces the investigators ability to estimate time of death with any degree of accuracy. Skeletonized material, depending on environmental

conditions, can exhibit an excellent state of preservation after thousands of years, while others can look ancient after only a few months. As a result, numerous attempts at quantifying the processes involved in bone aging, such as the loss of fat, protein and minerals, colour changes, surface texture changes, taphonomic changes, and chemical changes, have resulted in few applicable methods (Stewart, 1979: 70).

Many of the techniques which can be use to study bone for the purpose of aging have been outlined by Lunquist (1963). First, structural analysis can indicate age by estimating the degree of bone decalcification due to nitric acid. In environments with a relatively constant pH, bone shrinkage can be correlated to the time required for nitric acid to decompose the calcium phosphate lattice.

Bone weight and consistency can often be correlated with age. Old bones can be tested for carbonate by exposing them to hydrochloric acid. Production of "a lively foam" indicates considerable age, with younger bones producing only a slight reaction (Lunquist, 1963: 239). Fresh cross sections of bone can be checked for ultra-violet fluorescence, indicating the presence of organic ground compounds. As bones age, the loss of protein is represented in a fading of the fluorescence effect. X-ray structural analysis can check for the degree of formation of inorganic bone substance, or fossilization. In fossilized bone, carbonate is replaced by fluoride, and therefore, a rough estimation of age can be extracted by observing the amount of carbonate replacement (Lunquist, 1963: 238-243).

Finally, trace isotope analysis can determine the amount and type of radioactive isotopes present in bone and link it to different periods

of nuclear weapon testing. Advances in nuclear weapon technology during the 1950s and 1960s, combined with many test detonations, resulted in large amounts of radioactive isotopes being dumped into the atmosphere. Testing of nuclear weapons with different types of atomic fuel released different types of radioactive isotopes. These isotopes accumulate in living tissue, especially bone. As a result, it has become possible to determine if an individual died before 1950, between 1950 and 1960, or after 1960 by the radioisotope signature of their bones (Beattie, O., personal communicae).

Again, the majority of the techniques used to determine the age of bone cannot control for the high variability created by the environment. At best, analysis of skeletonized material will sometimes allow scientists to distinguish between recent and archaeological bone samples, or roughly place them in periods in history. Newly developed high resolution technologies have greatly improved the detail in which bone can be studied, but as of yet, the limits imposed on bone aging, due to variation in preservation, has made it difficult to apply these methods consistently to the determination of elapsed time since death.

CHAPTER THREE

DEOXYRIBONUCLEIC ACID (DNA) AUTODEGRADATION AS AN INDICATOR OF POSMORTEM INTERVAL

A lesson can be learned through close scrutiny of existing methods for determining time elapsed since death. Methods that directly, or indirectly, measure the decomposition process are subject to the same errors and variability that influence it. Effective methods for estimating the postmortem interval must be independent of changes directly associated with decay, exhibiting a relatively constant change over time, without wide variations resulting from fluctuations in environmental and climatic conditions. The only structures in the human body that fit these criteria exist at the molecular level.

DNA is an extremely stable molecule that is present in every nucleated cell. It exists as very long, double-stranded chains of nucleotides covalently linked. The purine and pyrimidine bases of one strand are bound by hydrogen bonds to complementary bases on the opposite strand and the bases within each strand are held together by phosphodiester bonds between an intermediary phosphate group on a sugar-phosphate backbone (Suzuki et al., 1986: 188). The two nucleotide chains run in a complementary antiparallel fashion approximately 20 Armstrongs in diameter and 34 Armstrongs in length between each complete helical turn (see Figure 1). This structure gives DNA considerable resistance to mechanical stress although it may be enzymatically or chemically denatured. Cutting enzymes, or nucleases, are manufactured and stored in the cell, and are released into the nucleus as required for proper cell activity. Nuclease production within the cell is continuous as each type of enzyme has a specific half-life, and is therefore limited in its period of activity once manufactured.

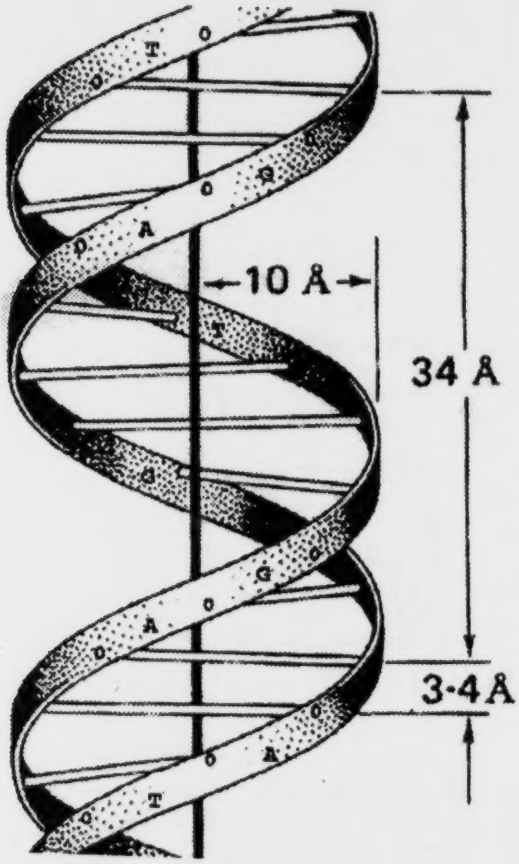

Figure 1: Shape and Size of the deoxyribonucleotide chain of DNA. (Adapted from Suzuki et al., 1986: 190)

Cell death leads to the eventual loss of integrity of the membrane bound enzyme storing vesicles. Within a few hours postmortem, autolytic enzymes and nucleases are released into the cytoplasm, resulting in the rapid digestion of cellular structures, including DNA. Nucleases begin to make cuts in the deoxyribonucleotide chain transforming it from a long single structure into multiple fragments. As the degradation process proceeds, the average size of the DNA fragments decreases proportionally. Eventually, the autolytic enzymes reach the end of their active life, slowing and finally stopping the digestive process.

Walter Bar and his associates (1988), have shown that the rates of postmortem DNA autodegradation are relatively constant, but tissue specific. In an attempt to quantify the postmortem stability of DNA, they found that, as the postmortem interval increased, their ability to recover DNA fragments of adequate size for Restriction Fragment Length Polymorphism (RFLP) analysis, decreased. Although the experiment was only conducted under controlled conditions, it did show the postmortem degradative activity of DNA lysing enzymes within the first twenty days after death. Bar et al. (1988), extracted DNA from tissue specimens of brain cortex, thyroid gland, lymphatic node, liver, spleen, psoas muscle, kidney and blood from 23 different bodies of known postmortem age. The samples were then run on electrophoretic gels and the amount of DNA extracted of adequate size for RFLP analysis was determined. The results showed that, as the postmortem interval increased, their ability to recover adequate DNA decreased. Each tissue type expressed different autodegradative rates, but a clear tendency to decrease autolytic activity over the postmortem interval was observed, representing the deactivation of the nucleases. Figures 2 through 6 represent the distribution of DNA (of required size) recovered from five different tissues plotted against postmortem time. They express the rate of DNA autodegradation during the postmortem interval, with the levelling of the best fit line indicating the slowing and eventual stop of the degradative process. The plot for blood (Figure 5), shows no DNA autodegradation over time, and is explained by the fact that white blood cells are relatively inactive metabolically compared to other cells like muscle or pancreatic tissue (Bar et al., 1988). The most metabolically active cells will autolyze first (Dr. Fiona Bamforth, personal communicae).

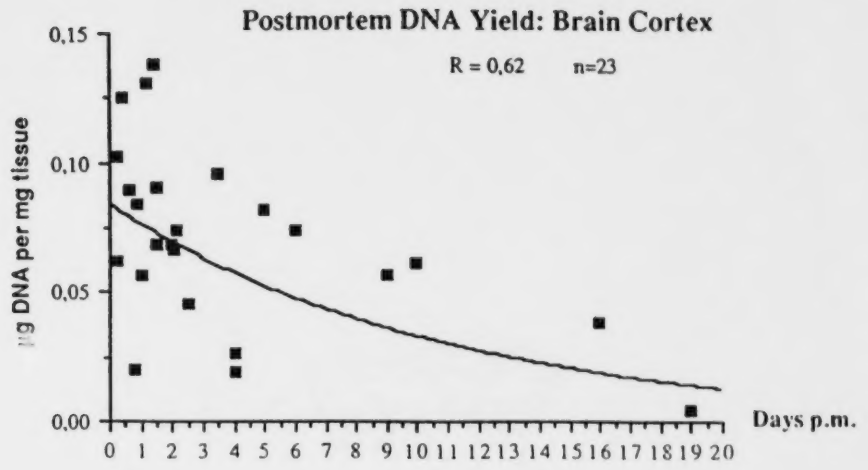
Postmortem DNA Yield: Brain Cortex
R = 0,62 n=23
µg DNA per mg tissue
0,15
0,10
0,05
0,00
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Days p.m.

Figure 2: Postmortem DNA Yield From Brain Cortex. A steady exponential decline over the postmortem interval was observed. (From Bar et al., 1988)

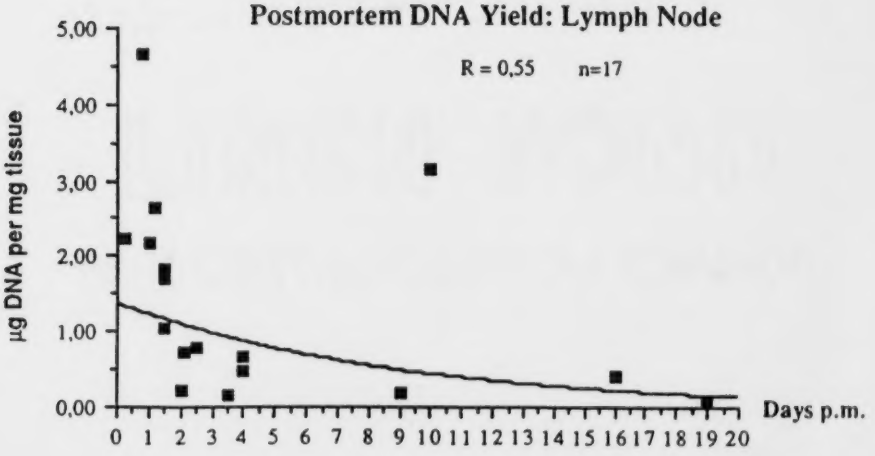

Figure 3: Postmortem DNA Yield From Lymph Node. Again, exponential decline over the postmortem interval was observed (From Bar et al., 1988).

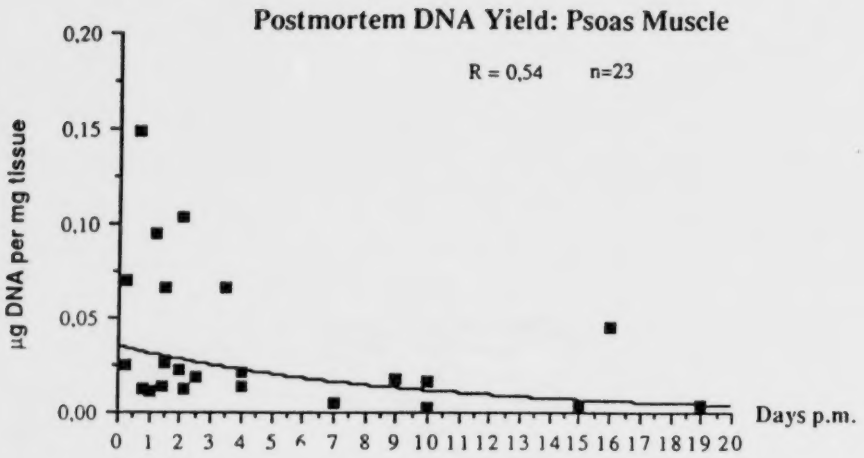
Postmortem DNA Yield: Psoas Muscle
R = 0,54 n=23
µg DNA per mg tissue
0,20
0,15
0,10
0,05
0,00
Days p.m.
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Figure 4: Postmortem DNA Yield From Psoas Muscle. Similar observation as with brain and lymph nodes(From Bar et al., 1988).

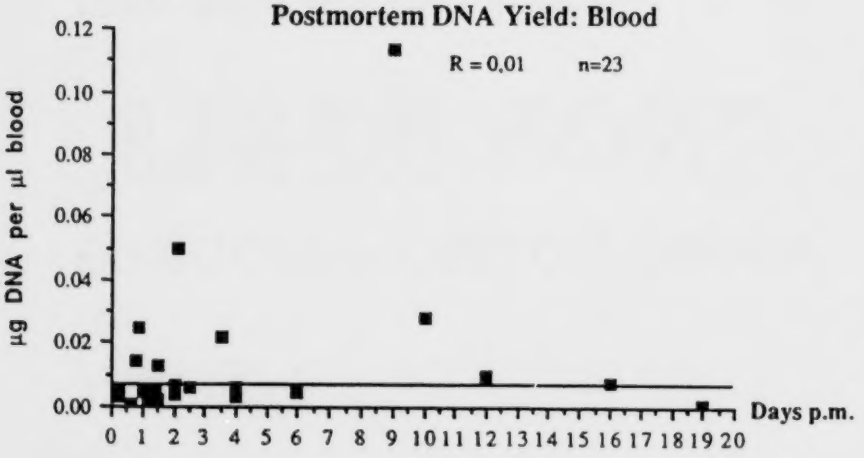

Figure 5: Postmortem Yield of DNA From Blood. DNA recovered over time remained constant as a result of the lack of autolysis(From Bar et al., 1988).

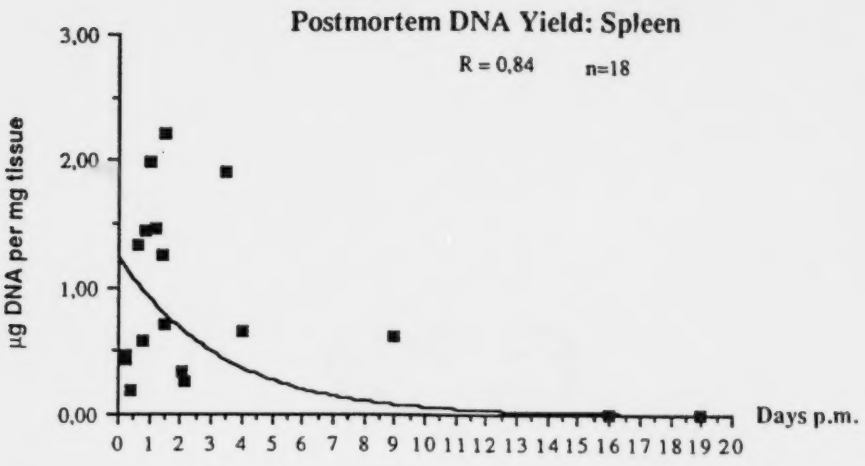

Figure 6: Postmortem DNA Yield From Spleen. Exponential decline similar to those of the other samples(From Bar et al., 1988).

A second, independent study, by Perry et al.(1988), attempted to correlate directly the postmortem rate of DNA autodegradation to the time elapsed since death. Their hypothesis states:

> As a body decays, the DNA molecules in all parts of the body are presumedly degraded by nucleases. These nucleases are presumedly released with other hydrolytic enzymes as cellular and subcellular membranes lose their integrity. As degradation continues, the average size of DNA molecules in the bone also decreases. If the degradation rate is similar enough in different individuals, and if the conditions where a corpse is found are known, and the amount of degradation is determined, the time since death might be estimated.
> (Perry et al., 1988)

Perry and his associates, extracted DNA from human rib and clavicle bone over a number of weeks postmortem; the DNA was run on electrophoretic gel, and the degree of fragmentation was noted. Results showed very little variation between individuals, the differences were accounted for by the type of bone used. DNA extracted from bone marrow continually degraded faster than DNA from compact bone.

Experiments were performed to test if variation in degradation rates could be produced by fluctuations in environmental conditions. DNA incubated at low humidity was found to degrade faster than DNA incubated in moist conditions, and an increase in temperature showed a corresponding increase in DNA breakdown. For all samples, DNA autodegradation slowed as the postmortem interval increased, and while it is difficult to interpret their plot of DNA fragmentation over time (Figure 7), an apparent levelling of the curve can be seen by about twenty days postmortem. Perry does mention that most of the DNA autodegradation occurred within six days postmortem, exponentially declining over the postmortem interval. No evidence for DNA autodegradation beyond the range eighty days postmortem is given.

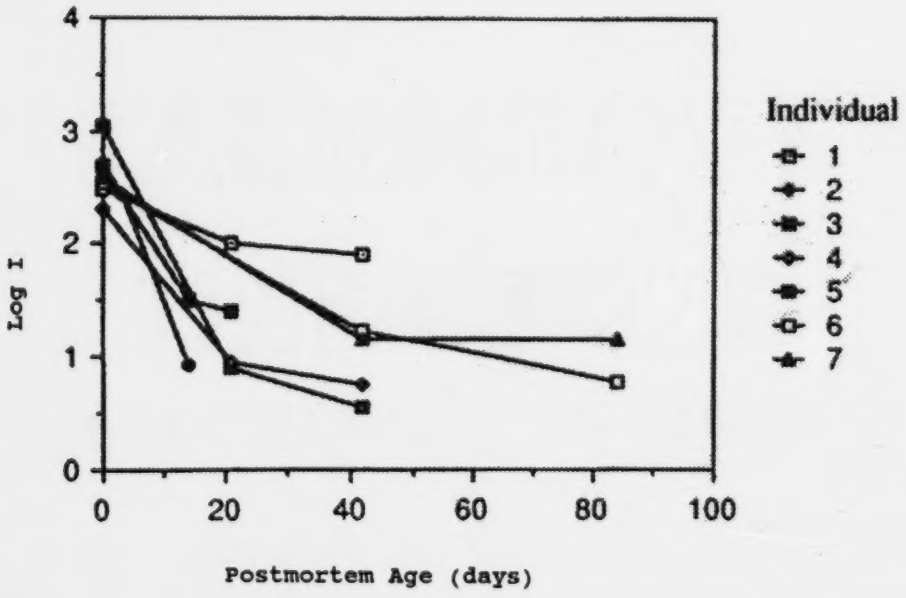

Figure 7: Display of DNA Integrity Over the Postmortem Interval. DNA Integrity is expressed as the log of the average fragment length. The exponential decline noted in the plots is moderated by using the logarithmic functions. (Adapted from Perry et al., 1988)

Close scrutiny of the work of Bar et al. (1988) and Perry et al. (1988), invites an interesting conclusion about the potential for using DNA autodegradation as an estimator of time since death. While Perry et al. have clearly tried to apply the technique to determine the postmortem period soon after death, the evidence supports the potential for a much more effective application for periods of greater duration. Levelling of the DNA integrity curves plotted by both Perry et al. (1988) and Bar et al. (1988), show the limits of the influence of decomposition processes on DNA breakdown. As the postmortem interval increases, a decrease in environmental influence on DNA breakage will invariably produce a more consistent method for the determination of time since death.

Nuclease activity is significantly influenced by environmental and climatic factors. Temperature and humidity can optimize or inhibit enzyme activity, thereby increasing or decreasing the rates of DNA hydrolysis, cell autolysis and decomposition. Evidence has shown, however, that DNA hydrolytic enzymes become inactivated as the postmortem interval increases (Perry et al., 1988; and Bar et al., 1988), therefore, within a relatively short period after death, the effects of environment on DNA autodegradation will slow. As the postmortem interval increases, the effect of the environment should become nominal. The work by Perry et al. (1988) and Bar et al. (1988), have shown that, as the size of the DNA molecule decreases, so does its chance of being broken (shown by the leveling of curves in Figures 2 through 7).

To reiterate, it is my hypothesis that in the early postmortem period, active autodegradation by nuclease action and mechanical

breakage will result in rapid fractionation of the DNA molecule. As the postmortem interval increases and the nucleases reach the end of their functional life, active autodegradation will cease. Further DNA breakage will only result from the combined effects of ionizing radiation, primarily from background levels of radon and solar radiation, and mechanical stresses due to climatic extremes. The average size of DNA fragments will continue to decrease, but at a considerably reduced rate. Eventually, the DNA fragments will be too small to be regularly affected by mechanical breakage, and only exposure to harsh chemicals or ionizing radiation will cause further disintegration. With the passage of time, mechanical breaks will occur, but with very low frequency, and single-strand nicks formed by ionizing radiation will accumulate, some leading to double-strand breaks, but very slowly. Both effects are guided by probability (ie. random), and therefore, over time will produce cuts at a relatively constant rate. The environmental conditions shown to influence decomposition should have little if any effect on this system.

In order to test this hypothesis it is necessary to establish whether DNA in the postmortem period (after deactivation of the hydrolytic enzymes) accumulates breaks at a constant rate. It is also necessary to establish whether this constant decrease in the average size of DNA fragments can be measured consistently and to an accuracy that will allow a usable estimate of time elapsed since death. Aquisition of environmental records is essential in order to account for climatic extremes in the test area and to prove that it is in fact possible to obtain climatic data detailed enough for this type of research. Tight guidelines must also be met regarding the age, sex, and antemortem condition of each sample used to ensure the differences in the quality and quantity of DNA extracted from each is the result of postmortem age, not individual variation.

CHAPTER FOUR

MATERIALS AND METHODOLOGY: THE NATURE OF THE SAMPLES AND DEVELOPMENT OF PROTOCOLS FOR THE QUANTIFICATION AND QUALIFICATION OF DEGRADED DNA FROM COMPACT BONE

NATURE OF SAMPLES

Six bovine right femora were collected from six different deposition sites within a one square kilometre area on a ranch near Cold Lake, Alberta, at 54 25' North, 110 17' West. All six specimens were located in areas with very similar localized environmental conditions. Each specimen was resting on the surface, fully exposed to the elements, on slight earthen rises ensuring that all would never have been completely immersed in water for any extended length of time.

The ranch owner, Derek Bamber, gave permission for the collection and use of all the material as well as supplied the farm records for the identification of each animal selected. These records included the birth and breeding history of each animal, the ear tag number and the date of death to the nearest month. It was common not to locate a dead animal immediately, therefore, the estimate of the date of death was the best that could be obtained. Each specimen was individually identified by the rancher and by its ear tag number (individual identification tag inserted into each cows ear) before it was collected. Only adult females that had died of natural causes were used for this work as a means of limiting criticism about the homogeneity of the sample group (see Appendix One for summary of sample group).

Selection of this farm as the collection site was made due to its unique location in relation to the Canadian Armed Forces Base, Cold

Lake, which is situated less than four kilometres from the area of the deposited specimens. As the base is one of the largest military airnbases in Canada, detailed climatic records have been kept for the past thirty years. Climatic data, including daily minimum and maximum temperatures, relative daily humidity, precipitation and daily, monthly and yearly norms were acquired for the sample collection area for the entire deposition period (Appendix Three). On final tally, the specimens ranged in age from 6 months to 18 years, with one sample of a 14 day old bovine femur being used to ensure quality control.

All six specimens were collected from their sites of deposition on the ninth of November, 1994. An entire right femur was taken from each, placed in a clean cellulose cardboard box, and marked with a sample number that correlated with the order collected and its original ear tag number. Each specimen was stored in its original cardboard box at 20 C in a secure office in the Department of Anthropology until processed for DNA extraction. The exception to this was the 14 day old control sample that was not collected until just prior to the start of the DNA extraction process, and was stored in a secure 4 degrees Celsius cooler in the Department of Plastic Surgery until the extraction began.

INTRODUCTION TO EXPERIMENTAL DESIGN

Developing an experimental design that could test the feasibility of using the quantity and condition of DNA as a measure of the time elapsed since death proved to be exceedingly difficult. While protocols for the evaluation of high molecular weight genomic and mitochondrial

DNA have been well established, few researchers have produced any proven methods for the extraction, purification and analysis of highly degraded DNA. Techniques found to be rigorous enough to process the exceptionally hard bovine compact bone could not ensure that artifactual degradation due to heating was avoided, and methods developed for the extraction of intact genomic DNA could not guarantee that DNA of small fragment lengths were not lost during the purification process. As well, it was found that standard methods for the quantification of DNA could not be used. The spectrophotometer, while being an excellent instrument for the quantification of non-degraded DNA, was found to give unrealistically high readings for highly degraded DNA when these concentrations were compared to those obtained from ethidium bromide quantity analysis for the same sample. For these and other reasons, considerable modifications to existing protocols, and in some cases, fabrication of new techniques, were required in order to complete this project.

BONE PROCESSING

Initially, attempts at processing the bovine femurs to extract the DNA using established protocols failed. The methods of Fisher et al. (1993), Hagelberg et al. (1991), Hochmeister et al. (1991) and Perry et al. (1988) for the extraction of DNA from human compact bone were all tried, modified and then abandoned due to their inability to process and extract DNA from the bovine femurs without generating artifactual degradation in the DNA extracted. The degree of grinding and crushing that was required to process the bovine femur samples generated excessive heat (greater than 80 degrees Celsius) that could lead to DNA degradation in the samples (Sambrook, Fritsch and Maniatis, 1989). As

one of the primary objectives of this research was to determine the average fragment length of DNA from each sample, zero tolerance could be given to any techniques that caused experimental artifacts. It was therefore necessary to develop a process that could crush bone of extreme hardness while generating little if any heat.

Extensive trial and error had shown that light utility grinding or crushing devices like the Waring blender (Perry et al., 1988) or the Tekmar Tisumizer (Fisher et al., 1993) required extensive lengths of time to crush the bovine femur. By the time the bone had been reduced to an adequate consistency for DNA extraction the temperature within the sample had increased, in some cases, enough to generate smoke (greater than 100 C) even while using liquid nitrogen as a coolant. Generally, what was discovered was that any method that could not reduce the bone to a powder within only a few blows of a mallet, or pulses of a blender, while the sample was frozen with liquid nitrogen, raised the sample temperature to an unacceptable degree. This created a substantial problem as, to my knowledge, no commercially available device existed that could perform this task.

After serious contemplation, it was decided that it would be necessary to fabricate the appropriate equipment using my own specifications and design. What resulted was basically a mortar and pestle that resembled a small bore piston and cylinder. Constructed primarily of cast iron and weighing in excess of 35 kilograms, the device could place a maximum of crushing force on a steel plunger 30 millimetres in diameter accelerating down a tube 0.5 metres long and of only a slightly larger diameter. Each bone sample had little if any play once placed in the tube, thereby not only stopping the bone from deflecting the crushing force, but also reducing the elastic rebound.

This substantially improved the plunger's ability to crush each piece of bone with the minimum number of compressions (see photograph, Figure 8). Typically, this machine could pulverize a 6 to 10 gram piece of bovine femur into 2 or 3 millimetre sized fragments in three successive blows, then be quickly disassembled to retrieve the fragments and be thoroughly cleaned and sterilized. After approximately a dozen trials using this device to crush 6 gram pieces of bovine compact bone, no significant increase in sample temperature was observed. Thus, this method was adopted as the primary means of processing my specimens. However, as it was required that the samples be completely powderized for the efficient extraction of the DNA, a second method was used to complete the bone processing procedure.

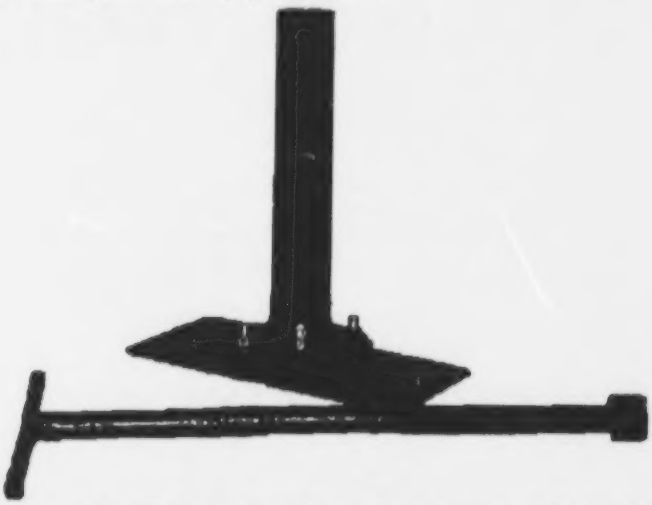

Figure 8: Photograph of Bone Crushing Instrument Fabricated to Process the Compact Bone from Bovine Femurs.

Once each 6 gram sample had been reduced to fragments of no larger than 3 millimetres, the sample was placed in a small stainless steel electric blender, completely emersed in liquid nitrogen and then ground momentarily, stopping well before the liquid nitrogen evaporated from the blender. This process was very efficient at completely powderizing the bone and never had to be repeated.

As a means of increasing the sample size, the seven samples were divided into two sets and extracted independently from each other 30 days apart. Set B was allowed to age undisturbed for an additional 30 days, then was processed, extracted and analyzed using the same methodology used in Set A. This substantially increased the sensitivity of this experiment by allowing the observation of any degradation that occurred within a 30 day period while the samples were in storage.

To summarize, all seven bovine femur samples were processed in two independent sets in the following manner: Each complete femur was crushed into pieces of approximately 5 to 10 grams using a large 2.0 kilogram mallet. Pieces estimated to be 8 to 10 grams or greater, from the medial, proximal diaphysis were selected and placed in clean cellulose paper bags, with the specimen number written on the outside. Each sample was then taken into the laboratory in a clean area, and using a Dremmel(TM) grinding tool, had the inner and outer tables of bone completely ground away along with the fracture planes of each fragment. Heat production was a concern, thus, every care was taken to ensure that the samples never became warm to the touch, or began to generate smoke (temperatures greater than 80 degrees Celsius). This processing not only removed all contamination present on the out side of the bone, but also ensured that only DNA protected from the direct action of the environment would be extracted. The remaining bone was thoroughly washed with deionized, sterilized water, allowed to air dry at room temperature, and then washed again with 98% ethanol. Once surface sterilization of the bone samples was completed, each was then completely immersed in liquid nitrogen for a minimum of 5 minutes, and then transferred into the crushing tube. Pulverization of the samples into 2 to 3 mm fragments was ensured with three quick compressions of the plunger. The device was then disassembled, the fragments collected

and transferred to a small electric blender filled with liquid nitrogen. A momentary pulse of the blender reduced the samples to a fine powder, completing the process.

Once this was completed, 6 grams of the powder was separated and placed in a 50 ml sterile polypropylene tube and then stored at -80 degrees Celsius until the beginning of the DNA extraction procedure. The exceptions to this were the two samples from the 14 day-old specimen; these were processed the same way, but just prior to the start of the DNA extraction.

It is important to note that during the entire procedure, all reasonable precautions were taken to ensure the samples were not contaminated by extraneous materials, or the powder from other samples. After each sample was processed, all the equipment was thoroughly cleaned and sterilized by washing it twice with sterile deionized water and once with 98% ethanol. New, sterilized, polypropylene tubes were used for each sample, and clean latex examination gloves were worn for each process. Laboratory benches were completely cleaned of bone powder and the bone waste was disposed of to ensure that the work area remained free of contamination sources.

DNA EXTRACTION & PURIFICATION

A number of well established protocols for the extraction and purification of DNA from compact bone were carefully examined, and in some instances, tested, while developing a methodology for this research. Some of these were eliminated quickly due to their specific

application to high molecular weight DNA, others abandoned later due to problems associated with DNA yield, or quality control. The method of Perry et al. (1988), Hochmeister et al. (1991) and Hagelberg et al. (1991), for example, all used 0.5 molar EDTA (a strong anion chelator) to decalcify completely the bone samples prior to DNA extraction. This process took between three to five days on average to complete, and is known to cause a substantial decrease in the overall yield of the DNA extracted (Fisher et al., 1993). Although during the decalcification process the samples were kept at 4 degrees Celsius, theoretically decreasing the chance that further DNA degradation would occur, the results of the research would be skewed by the artifactual loss of yield. It was decided, therefore, not to include decalcification in the extraction procedure.

A second serious source of sample quality and quantity artifacts that were avoided in the DNA extraction protocol was the use of multiple phenol extractions for the removal of excessive protein contamination. While the well established protocols of Fisher et al. (1993), Hagelberg et al. (1991), Hochmeister et al. (1991) and Perry et al. (1988) all advocated using multiple phenol/chloroform extractions, it was discovered that phenol selectively eliminates up to as much as 25% of small fragment length DNA with each successive extraction (Sambrook, Fritsch and Maniatis, 1989). In establishing a methodology for the research presented here, this source of error could not be tolerated. While, reducing the number of phenol extractions increased the presence of protein contamination in each sample, it was decided that avoiding the selective loss of low molecular weight DNA was more important. For this reason, the extraction protocol only included one phenol extraction. In fairness to the protocols of the researchers mentioned above, it should be noted that in a study involving the extraction of

non-degraded DNA, the loss of low molecular weight fragments would be irrelevant. It only becomes an issue when attempting to determine the overall quality of a highly degraded sample.

The following is a step by step account of the protocol developed for the extraction of degraded DNA from compact bone. The six, 6 gram samples of powderized bovine compact bone were divided into 1 gram portions and placed in ultraviolet light sterilized 50 ml polypropylene tubes. A 1 gram sample of the 14 day old sample was similarly prepared as a control. 2 ml of prewarmed (56 degrees Celsius) extraction buffer was added to each tube. The extraction buffer included: 0.01 molar Tris (hydroxymethyl) aminomethane (Tris), 0.01 molar disodium EDTA-$2H_2O$, 0.1 molar sodium chloride, 0.039 molar dithriothreitol (DTT), and 100 microlitres Proteinase-K (20 mg/mL). The tubes were then incubated at 56 degrees Celsius for two hours with intermittent shaking, and 10 hours without agitation. An additional 100 microlitres of Proteinase-K (20 mg/mL) was added to each tube and incubated at 56 degrees Celsius for another 3 hours.

The samples were subsequently transferred to sterile glass tubes and extracted once with an equal volume of phenol/chloroform and isoamyl alcohol (25:24:1). Each was centrifuged for 15 minutes at 2000G to ensure separation of the layers, and the aqueous phase of each tube was transferred to another sterile glass tube where it was extracted twice with water saturated ether. Traces of ether were removed by warming the samples to 56 degrees Celsius while blowing a stream of nitrogen gas across the top of the tubes. 2.2 volumes of "ice cold" (-20 degrees Celsius) ethanol and 0.1 volumes of 3 molar sodium acetate were added to each tube, precipitated at -20 degrees Celsius for 8 hours and then centrifuged at 2000G for 15 minutes. The retentate was washed three

times with 2 mL of TE buffer composed of 0.01 molar Tris and 0.001 molar $Na_2EDTA\text{-}2H_2O$ at a pH of 7.5. One-hundred microlitres of the same TE buffer was added to each pellet and then stored at 4 degrees Celsius until prepared to gel electrophoresis.

DNA VISUALIZATION

The visualization of the DNA samples was accomplished through electrophoresis in ethidium bromide stained neutral 2% agarose gels. The gels were prepared by boiling 3.4 grams of laboratory grade agarose in 170 millilitres of TBE (Tris-borate). Once the agarose fully liquified, the solution was removed from heat and 1 microlitre of ethidium bromide (10 mg/ml in water) was added for every 10 millilitres of TBE giving a final ethidium bromide concentration of 5 micrograms per millilitre in the agarose/TBE solution. The solution was allowed to cool to 50 degrees Celsius while being continuously stirred, and then was poured into a gel plate and allowed to setup.

As salt concentrations in the DNA samples were a serious source of concern due to sodium's ability to compete selectively with ethidium for DNA binding sites (Sambrook J., E.F. Fritsch and T. Maniatis, 1989), the DNA samples were prepared in the following way: Each sample was taken out of 4 degree Celsius storage and washed 3 times with -20 degree Celsius 70% ethanol in order to eliminate accumulated salts. The pellets were then resuspended in 100 microlitres of TE buffer, and thoroughly dissolved into the solution. Quantities of each sample (summarized in Table 1) were transferred to sterile 2 millilitre Eppendorf tubes along with 10 microlitres of loading dye (0.25% bromophenol blue, 0.25% xylene

cyanol FF and 15% Ficoll type 400m; Pharmacia, in water) and small amounts of TBE suspension buffer. The TBE buffer was used to ensure that all wells on the gel would have equal volumes of solution loaded (60 microlitres). Each tube was placed in a heat chelator and warmed to 65 C to separate the DNA double helix and reduce secondary DNA chain interactions, and then immediately loaded onto the gels. A Lambda HindIII marker was prepared and loaded on each gel in a similar manner. Figure 9 summarizes the preparation of the agarose gel.

	Sample (days)	Quan.Loaded (microlitres)
SET A.		
	14	50
	180	50
	330	50
	660	50
	3650	50
	4380	50
	6570	50
SET B.		
	44	20
	210	20
	360	20
	690	30
	3680	40
	4410	40
	6600	40

Table 1: Quantities of Each Sample Loaded Onto Each Electrophoretic Gel.

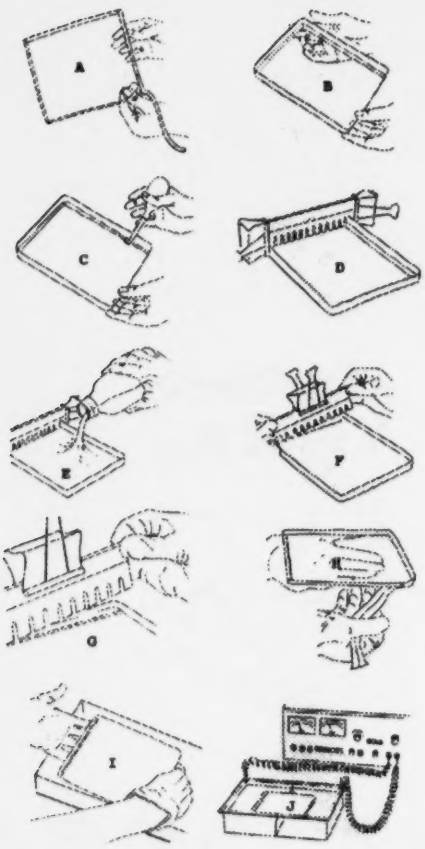

Figure 9: Summary of Electrophoretic Gel Preparation (Adapted from Sambrook, Fitsch and Maniatis, 1989).

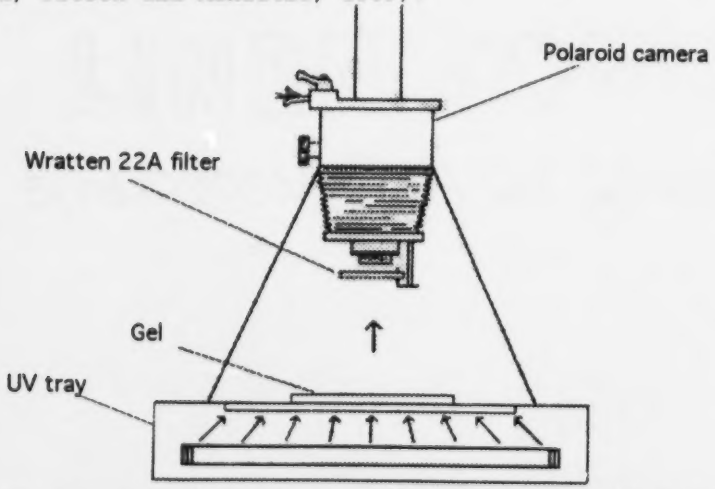

Figure 10: Summary of Electrophoretic Gel Photograph Process (Adapted form Sambrook, Fitsch and Maniatis, 1989).

Each gel was run at 100 volts for the first 15 minutes and then at 20 volts for 24 hours. After this, the gels were photographed on a Chromato-vue(TM) Trans-Illuminator using a Polaroid(TM) black and white film and a Kodak Wratten 22A filter. Figure 10 is a schematic of the photography of electrophoretic gels by ultraviolet illumination.

Initially, my experimental design included the transfer of the DNA from each gel to nitrocellulose membranes in preparation for hybridization. Unfortunately, while the Southern Blot transfers (Southern, 1975) were quite successful, hybridization with a collagen type-I probe was not. This short-falling eliminated my ability to distinguish between degraded bovine DNA and bacterial/algal contamination, but was unavoidable due to cost restrictions. As a result, analysis of each sample was completed using the Polaroid(TM) photographs, and included the extraneous influences of small quantities of high molecular weight microbial contaminates. The protocol used for the transfer of the DNA from the agarose gels to solid mediums is included here only for posterity.

The DNA from the electrophoretic gels were transferred to a solid medium in the following way: Each agarose gel was denatured in 1.5 molar sodium chloride and 0.5 molar sodium hydroxide for 45 minutes and subsequently rinsed three times with sterile, deionized, water. They were then neutralized in 1 molar Tris (pH 7.4) and 1.5 molar sodium chloride for an additional 45 minutes, changing the neutralization solution after 30 minutes. 2 X SSC (3.0 molar sodium cloride, 0.3 molar trisodium citrate) was used to wash the gels just prior to placing them in contact with the nitrocellulose membrane.

The transfer apparatus was prepared by cutting one piece of nitrocellulose membrane and one piece 3MM(TM) paper to the size of the gels. One piece of 3MM paper was also cut to the length of the gels, but approximately 3 times the width. This paper was laid over a support approximately the size of the gel and allowed to drape into copious amounts of 15 X SSC transfer buffer. The gels were placed on top of this and the nitrocellulose membrane placed carefully on top of the gel, ensuring no air bubbles were trapped between. The 3MM paper cut to the gel size was then added on top of the membrane, and approximately a 10 centimetre bundle of flat paper toweling was placed above. A glass plate was set atop the toweling, and finally, a 500 gram weight placed on top of this. All was wrapped in clear plastic and allowed to transfer overnight. Figure 11 is a schematic of the Southern Blot apparatus used.

Within 12 hours the DNA stained with ethidium bromide could be seen on the nitrocellulose membrane when placed over an ultraviolet light illuminator tray and none could be seen in the agarose gel, ensuring that the DNA transfer was complete. The membrane was vacuum baked at 80 degrees Celsius for 2 hours to fix the DNA, and then was stored at room temperature until the attempted hybridization.

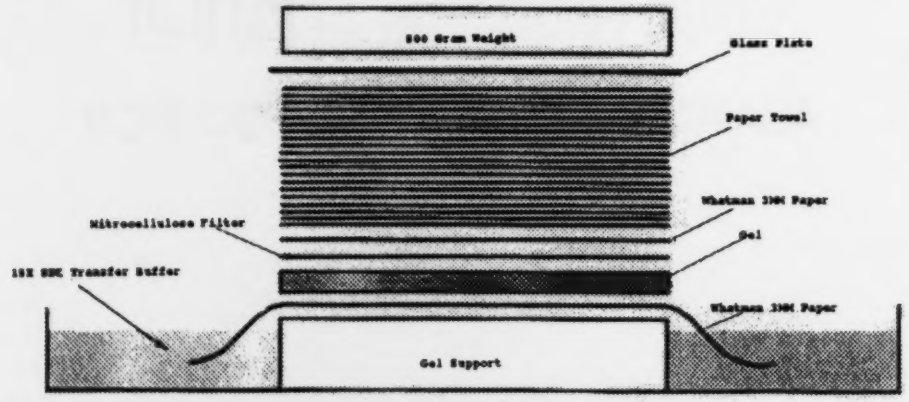

Figure 11: Southern Blot Apparatus (Adapted from Sambrook, Fitsch and Maniatis, 1989).

Hybridization was attempted using a collagen type-I probe labelled with phosphoros-32(alpha) via in vitro nick translation. This procedure was completed by the laboratory head, Dr. Aziz Garhary, but was unsuccessful. The blots used were destroyed in the attempt, and further hybridization attempts were not possible. It should be noted that the protocol used was that designed for RNA hybridization, and may have been the cause of the failure.

Collagen type-I is a large probe of approximately 4600 base pairs. When probing for RNA in a sample, the solution will contain many thousands, if not millions, of RNA copies of this large molecule. Thus, if stringent conditions are met, extremely well labelled bands can be expected. When probing for the DNA copy of collagen type-I, however, only one copy of the gene exists per cell, and if the DNA is severely degraded, as in this case, it becomes almost impossible to bind a large molecular weight probe to a single highly degraded gene. To hybridize highly degraded genomic DNA successfully it would be necessary to use protocols established specifically for probing DNA. It would also require a probe of a small size specific for a genomic site with many thousands, if not millions, of copies. Sequences contained within the centromeric region of the genome, for example, would most likely suit these requirements.

QUANTIFICAION OF DNA

Initial attempts to quantify the DNA samples using spectrophotometric analysis was unsuccessful. Spectrophotometric readings taken for samples and then compared to the same samples

visualized on ethidium stained agarose gels showed major inconsistencies; estimates of many hundreds of micrograms from the spectrophotometer could be visually estimated to be only nanograms when displayed with ethidium fluoresence. As a result, research into the theory of spectrophotometric analysis turned up the fact that at the wavelength of 260 nanometres, the appropriate wavelength for estimating the quantities of high molecular weight double-stranded DNA, the long DNA strands interact with one another reducing the amount of absorption that would be expected knowing the absorption of the four bases that make it up. Therefore, the shorter the lengths of DNA present in a solution the greater the amount of light that is absorbed and the higher the concentration reading (Berger, S., 1987: 33-61). When estimating concentrations for highly degraded DNA the spectrophotometric readings are better estimates of the number of DNA fragments than they are of the total quantity of DNA present. Thus, it was essential for the successful completion of this research that a new method for estimating the quantity of highly degraded DNA in samples be developed.

Visualization of the DNA samples was already required in order to estimate the quality of the DNA being extracted. I pondered the idea, therefore, of using the relative densities of ethidium bromide fluorescence in the agarose gels, calibrated to a known amount, as an estimate of total quantity. Theoretically, if the rate of ethidium binding to the duplex DNA can be controlled for all samples on a given gel, and the DNA concentration is known for at least one of the samples, comparing the ethidium fluorescence of the unknown samples to the known sample should give an estimate of DNA quantity. Because the Lambda HindIII marker is loaded on each gel in measurable quantities and separates into distinct bands during electrophoresis, it could be easily used for this purpose. This formed the basis of the technique that was

finally developed.

The following is a point by point account of the procedure developed for estimating the quantity of highly degraded DNA from ethidium bromide stained agarose gels:

1. A quantity of Lambda HindIII marker DNA was loaded onto the electrophoretic gel stained with ethidium bromide along with a documented volume of each sample being measured.

2. The electrophoretic gel was run, and a Polaroid(TM) photograph was taken as per the previously described protocol.

3. Using the National Institutes of Health's (NIH) gel plotting software included with Image 1.41 VDM (TM), the Polaroid(TM) images were digitalized using a high reslultion flat-bed scanner and a density analysis plot for each column was made. Each plot resulted in a comparable reading of the amount of ethidium binding DNA in each sample.

4. Using the known amount of Lambda HindIII marker loaded on each gel, the quantity of DNA that would be found in each of the definable bands was determined. This was done by calculating the percentage of DNA found in each size band relative to the total DNA in the Lambda ring. This equation can be expressed in the following way:

$$a=xy(v/t) \qquad 1$$

where:

x = volume of Lambda HindIII loaded (microlitres).
y = concentration of Lambda HindIII DNA in solution (micrograms/microlitre).
t = total size of Lambda DNA molecule(base pairs).
v = size of each Lambda HindIII fragment(base pairs).
a = amount of Lambda DNA found in each band (micrograms).

For example, the Lambda ring is composed of 48502 base pairs and the smallest Lambda HindIII fragment is 564 base pairs, which is 1.1628% of the total ring. Thus, in any Lambda HindIII marker column the 564 base pair band will contain 1.1628% of the total Lambda DNA loaded. If 15 microlitres of Lambda HindIII DNA is loaded, the 564 base pair band will contain 87.2 nanograms of DNA if the initial solution concentration was 0.5 micrograms per microlitre.

5. From the NIH plot of the Lambda HindIII column, the area under the curve for each definable size peak was determined in pixels, the number of pixels per band being equal to a given DNA quantity calculated for that band. Thus, for each gel, a microgram per pixel value was obtained. The area under the curve from the density plots for each unknown sample was then calculated in pixels. This pixel amount was used to calculate the amount of DNA in the unknown sample, in micrograms, by multiplying that value by the micrograms per pixel value determined for that gel.

Figures 12 through 27 represent the density plots for each Lambda HindIII marker and samples. Figure 12 and 20 are the Lambda plots used to calibrate the microgram/pixel quantity. The rest give a pixel amount for each unknown sample used to determine the final DNA quantity.

6. Finally, the quantity of DNA found in each gel column, in micrograms, was calibrated using the percentage of total sample loaded and the amount of bone originally processed, to produce a value for the amount of DNA extracted (in micrograms) per gram of bone. The equation used to calculated this amount is as follows:

$$y=(x(t/v))/r \qquad 2$$

where:

t = total sample extracted from bone (microlitres).
v = quantity of sample loaded onto gel (microlitres).
r = total weight of original bone sample (grams).
x = amount of DNA found in each gel column (micrograms).
y = quantity of DNA per gram of bone processed (micrograms).

These results are discussed later in Chapter 5.

The method developed here for estimating quantity of highly degraded DNA is a logical technique if a certain condition is met. As the quantity of DNA is being indirectly measured via the analysis of ethidium bound to it, the conditions for ethidium binding to the DNA must be relatively equal in all the samples in order to calibrate the system properly. For example, as it is commonly known that excessive amounts of sodium in a sample competes with ethidium for binding sites on the DNA complex, differing concentrations of salts in the samples will result in variable ethidium binding. If this occurs, the calibration will be erroneous and will result in false quantity estimates. Also, ethidium bromide is known to bind coiled and supercoiled DNA with a higher affinity than DNA in a non-coiled form. Attempts were made in this research to control for the amount of salt present in each sample by washing each with 70% ethanol 3 times before loading the electrophoretic gels and the samples were heated to 65 degrees Celsius just prior to loading in order to limit any differences in DNA coiling and supercoiling between the samples. Of course these measures could only limit major differences in ethidium binding and could not account for all of the numerous interaction variables.

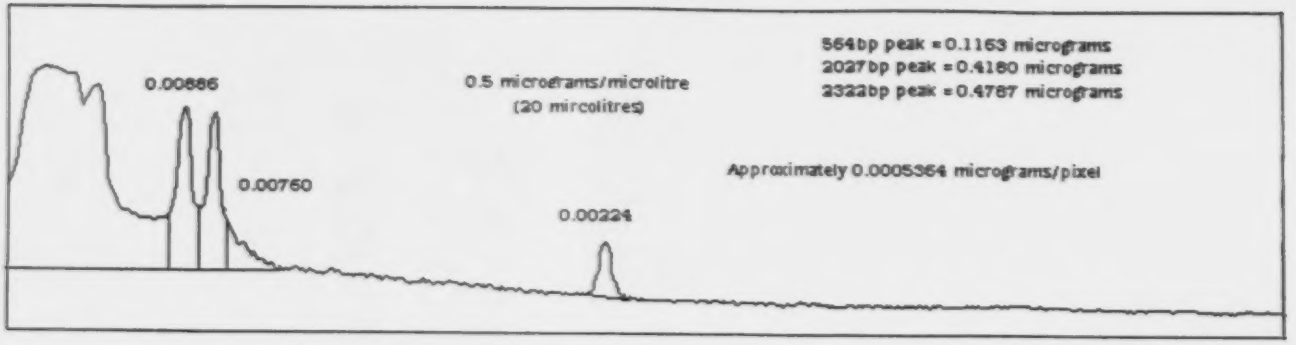

Figure 12. Density plot of Lambda HindIII marker form set A.

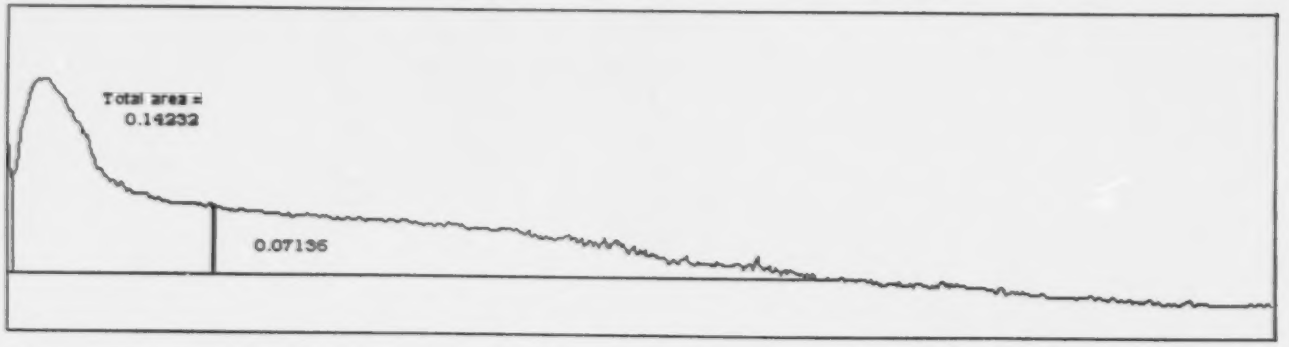

Figure 13. Density plot of the 14 day old sample from set A.

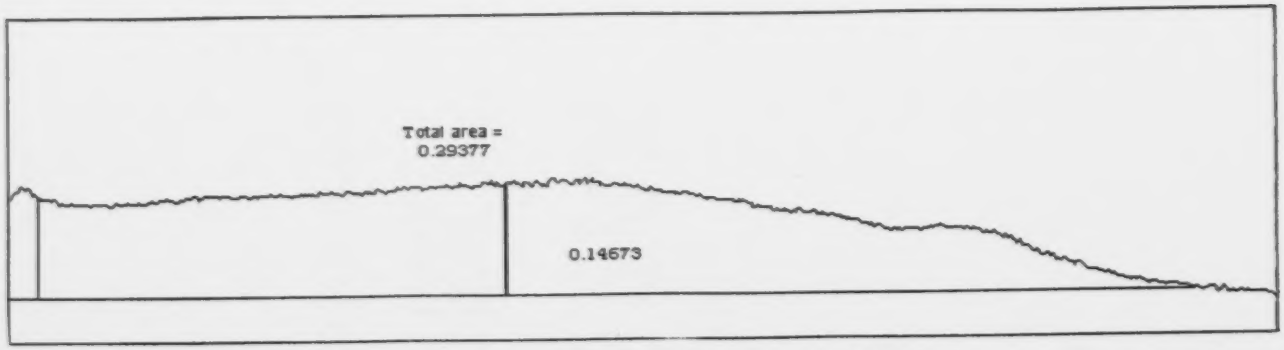

Figure 14. Density plot of the 180 day old sample from set A.

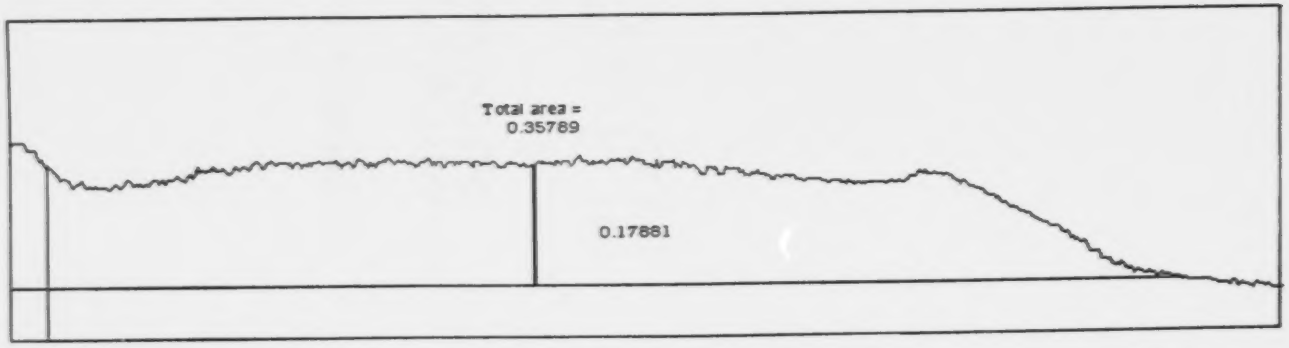

Figure 15. Density plot of the 330 day old sample from set A.

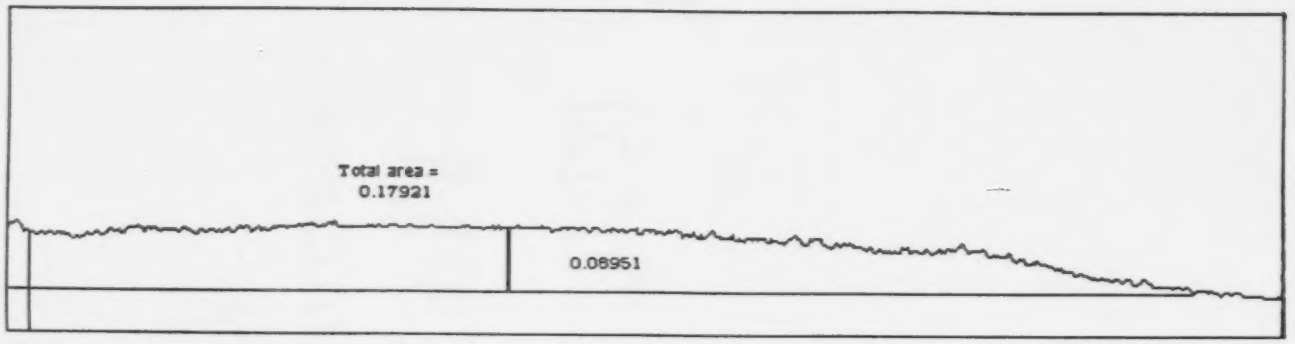

Figure 16. Density plot of the 660 day old sample from set A.

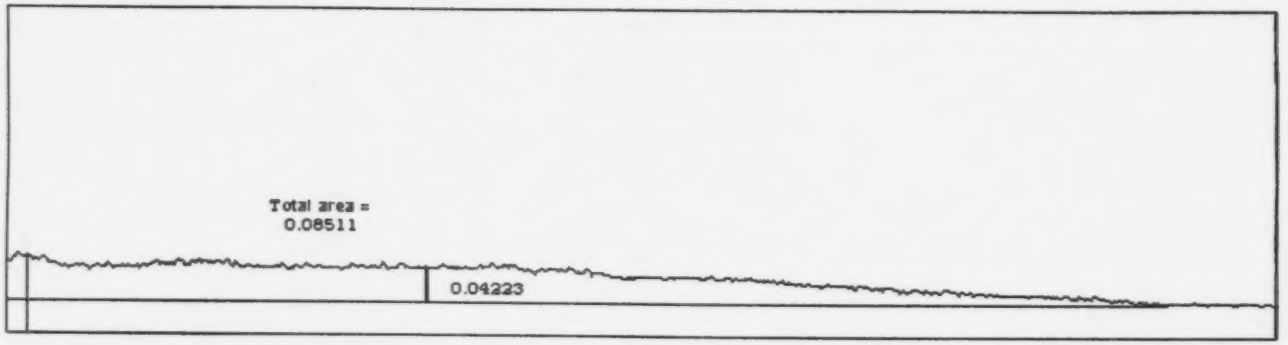

Figure 17. Density plot of the 3650 day old sample from set A.

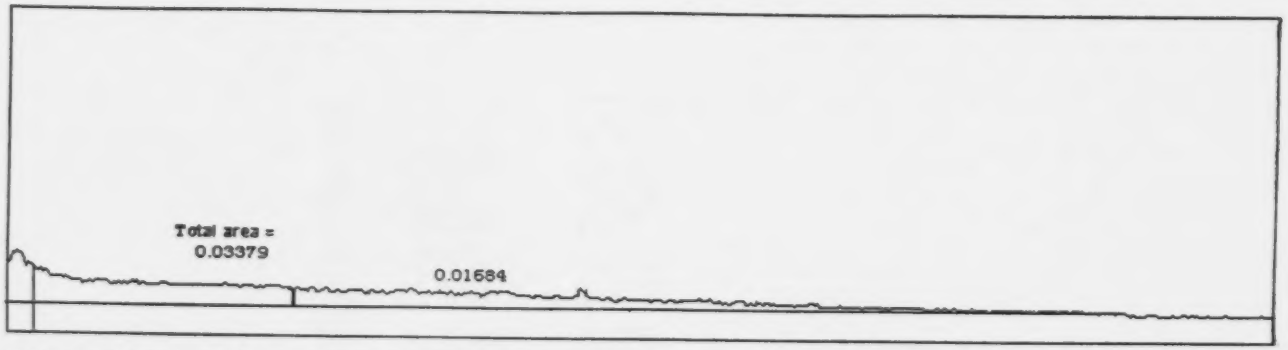

Figure 18. Density plot of the 4380 day old sample from set A.

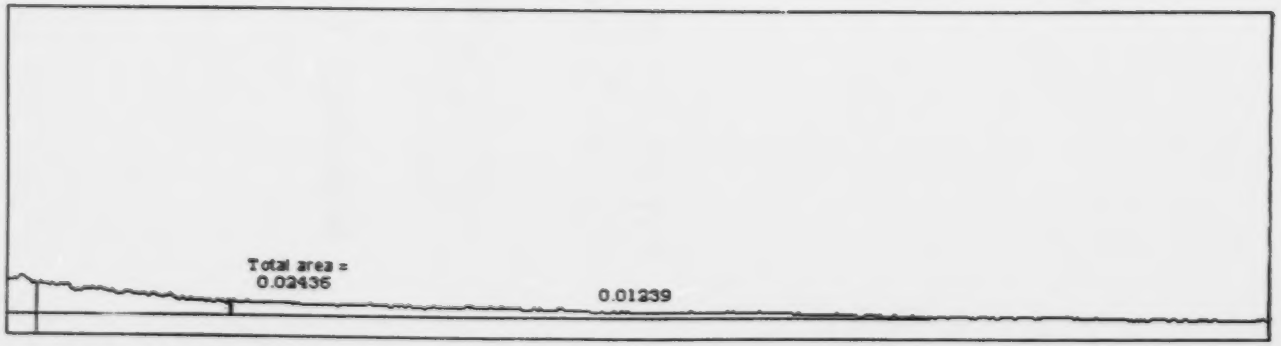

Figure 19. Density plot of the 6570 day old sample from set A.

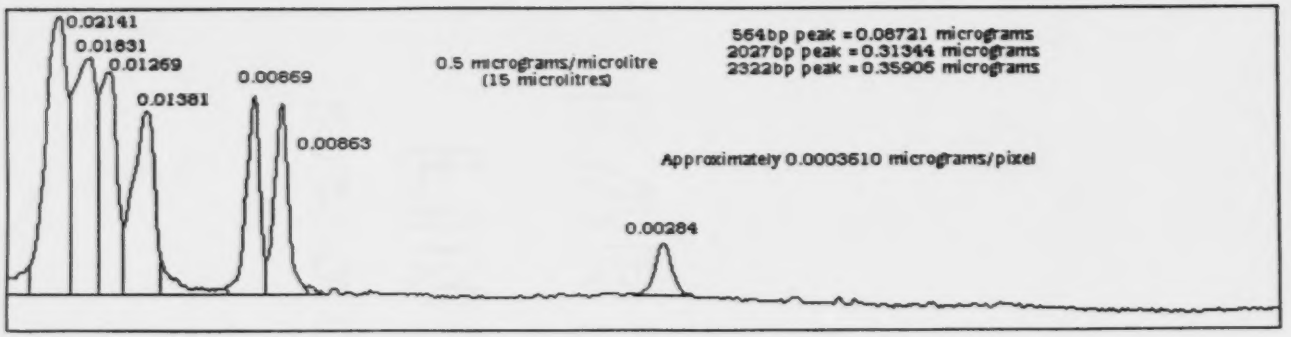

Figure 20. Density plot of Lambda HindIII marker from set B.

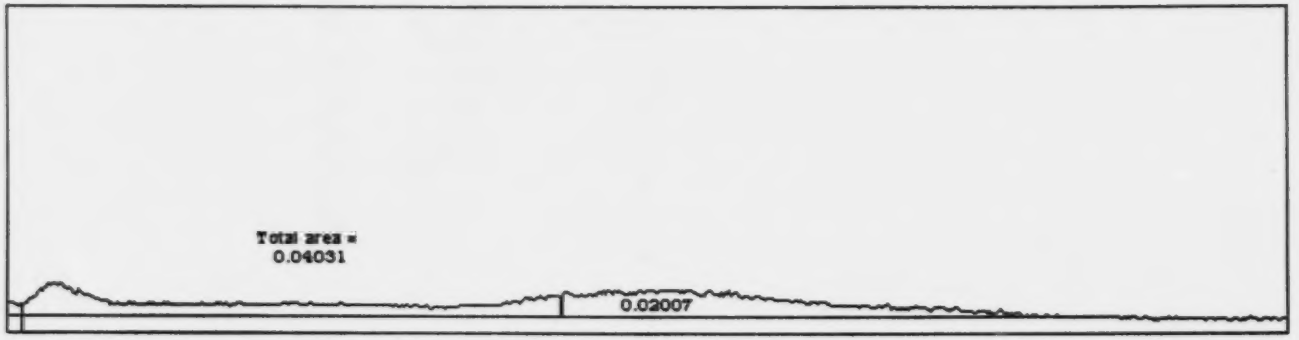

Figure 21. Density plot of the 44 day old sample from set B.

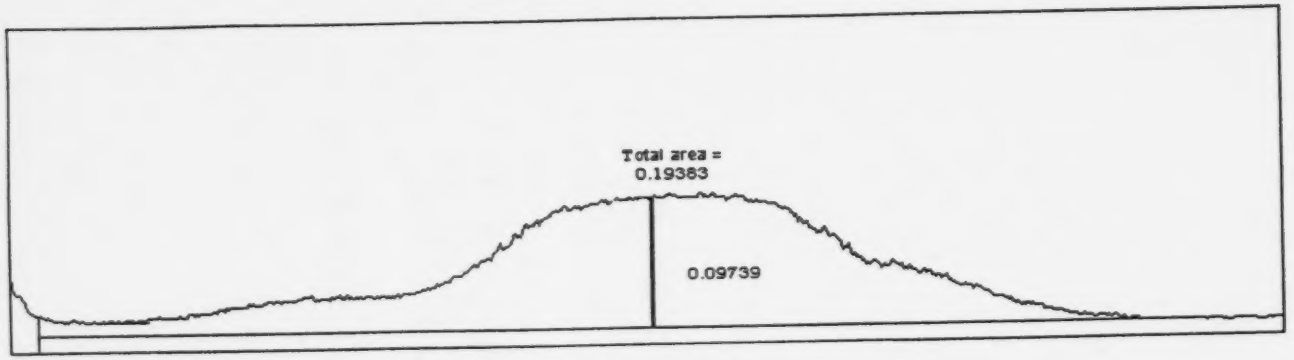

Figure 22. Density plot of the 210 day old sample from set B.

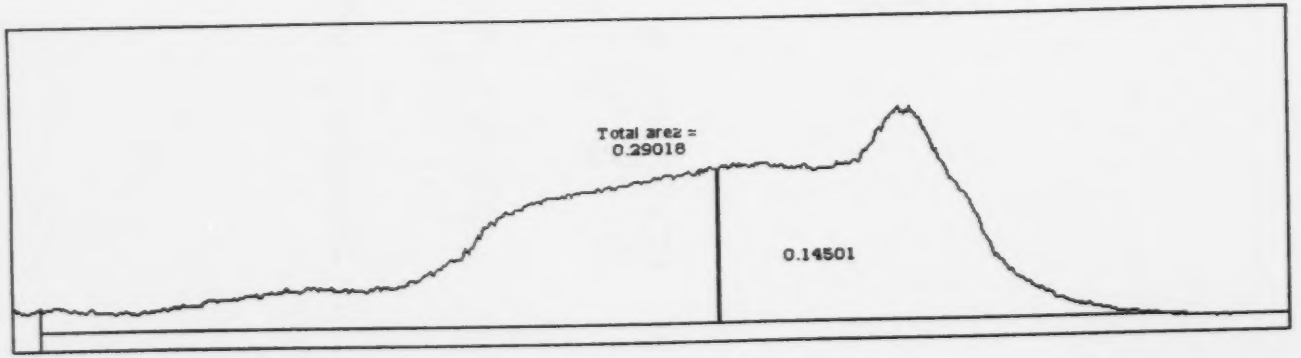

Figure 23. Density plot of the 360 day old sample from set B.

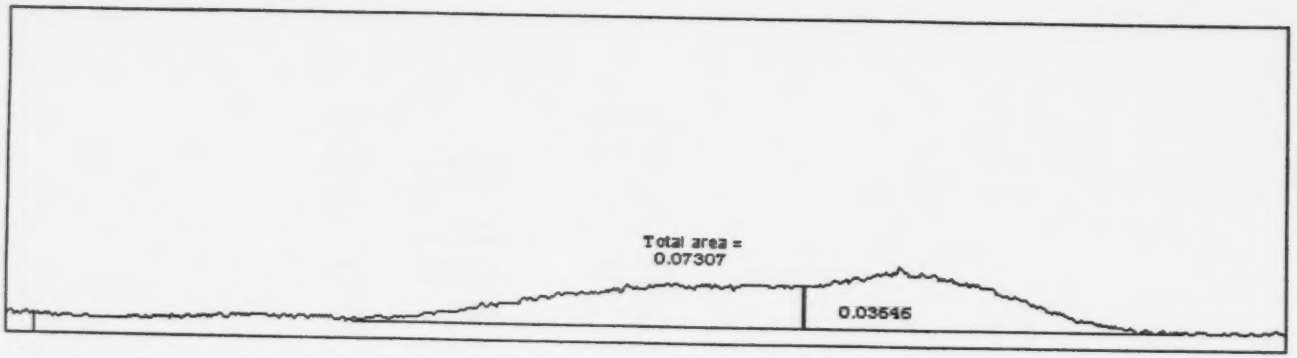

Figure 24. Density plot of the 690 day old sample from set B.

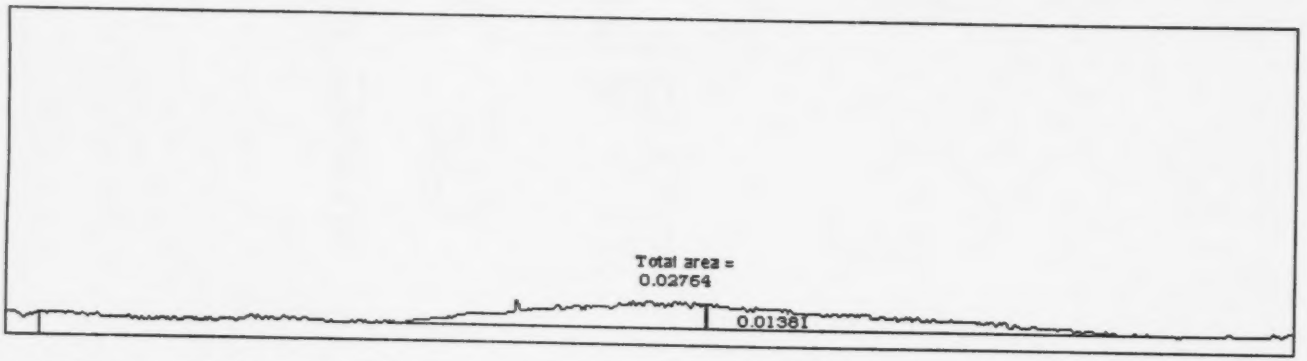

Figure 25. Density plot of the 3680 day old sample from set B.

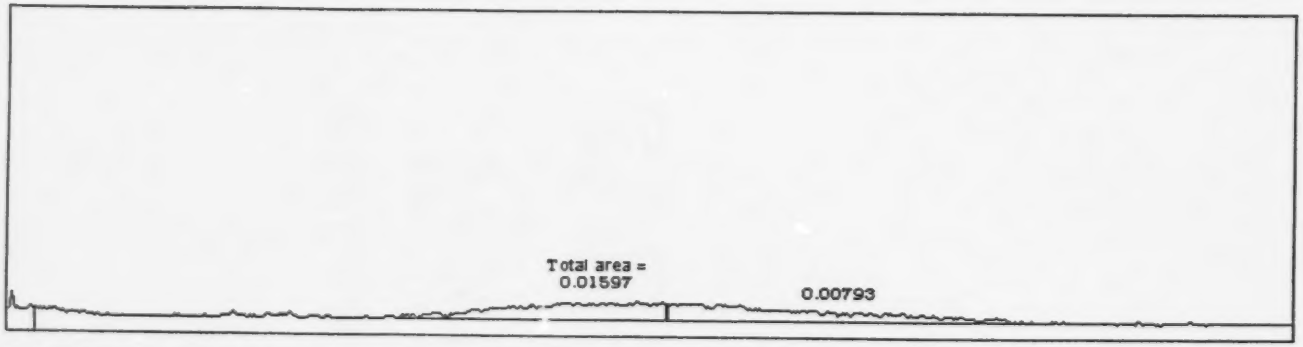

Figure 26. Density plot of the 4410 day old sample from set B.

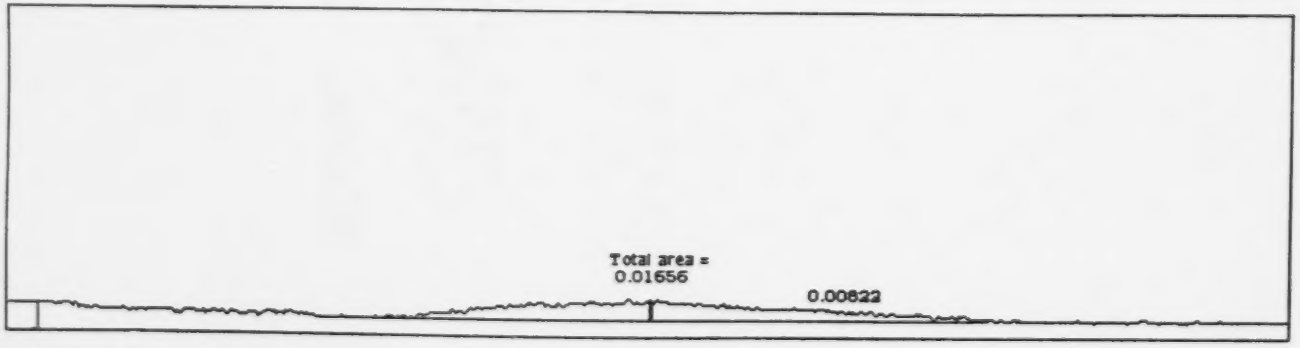

Figure 27. Density plot of the 6600 day old sample from set B.

QUALIFICATION OF DNA

The qualification of the DNA for the purpose of this research basically involved the determination of the DNA postmortem condition for each of the samples. As these samples are naturally degraded, no definable bands were expected, nor were they found, on the electrophoretic gels. Density plots for each sample showed a broad streak, with essentially a wide variation in fragment lengths. For this reason, determination of the average DNA fragment length was the best estimate for the degree of degradation found. By calculating a single value that was representative of the overall condition of the DNA in each sample, a quick and relatively error free method of comparing the level of postmortem DNA degradation was produced.

Electrophoretic gels function to separate DNA molecules based on their ability to migrate through a porous medium within an electric field. DNA, which is a negatively charged molecule, is pulled toward a positive anode in a strongly conductive fluid medium. When the DNA is loaded into agarose the electorphoretic attraction pulls the DNA into a gel that acts as a very fine sieve. Because all DNA molecules carry the same negative charge, only the relative size and complexity of the molecule will determine the speed at which it migrates through the agarose. The smaller and less complex the molecule, the faster it travels; the larger and more complex the molecule, the slower it travels. The use of this system for this research allowed an easy way of separating the degraded DNA molecules in a systematic fashion, and when run along with a DNA marker like Lambda HindIII, an accurate determination of the size of the DNA molecules found in each sample could be made.

The DNA from each of the seven samples was loaded into a 2% agarose gel and electrophoresed for 24 hours at a very low voltage. The high concentration of the agarose and the low voltage used, ensured a maximum level of sensitivity for visualizing the differences in fragment sizes. A Lambda HindIII marker was run on each of the agarose gels electrophoresed.

Using the National Institutes of Health's gel plotting software, a density plot was obtained for each of the columns on the electrophoretic gels (Figure 12 through 27). For each unknown sample the area under the plotted curve was calculated (in pixels) then divided by two. This value, being representative of half the area under the curve, was used to position a line on the plot dividing that area in to two equal parts. The position of this line relative to the distance migrated, once calibrated to fragment size, determined the average fragment size for each of the unknown DNA samples. Calibration was accomplished using the known fragment sizes and migration distances for the Lambda HindIII sample ran on each gel.

The distance each Lambda HindIII size band migrated on the gel was carefully measured and documented (Table 2). Using these data, plots of distance migrated verses the log of the fragment size were produced (Figure 28 and 29). The linear section of graphs being extrapolated, if necessary, to include the entire range of the distances migrated for all the unknown samples. The distance of each line placed on the density plots for the unknown samples to the migration starting position were then taken and documented. Then by positioning these distances on the Lambda HindIII plot of migration distance verses the log of the fragment size (Figures 28 and 29), each distance could be correlated to a given fragment length. The distances of each of the lines on the density

curves are representative of the average distance migrated by all the fragments in each sample, therefore, comparing it to the Lambda HindIII calibration curve allowed the determination of the average fragment length for each unknown sample (Table 3).

SET A.

Distance (mm)	Fragment size (bp)
7.18	23130
11.82	6557
16.88	4361
33.44	2322
38.94	2027
112.36	564

SET B.

Distance (mm)	Fragment size (bp)
9.92	23130
15.58	9416
19.42	6557
26.48	4361
46.76	2322
51.88	2027
123.74	564

Table 2. Migration Distances for Lambda HindIII Markers on Set A and set B.

Postmortem age of Bone (days)	Distance Migrated (mm)	Average Fragment Size (b.p.)
SET A.		
14	39.40	2000
180	93.56	790
330	98.82	710
660	94.22	780
3650	79.14	1000
4380	54.90	1700
6570	42.66	1900
SET B.		
44	104.22	800
210	121.08	600
360	133.70	480
690	150.28	360
3680	131.00	500
4410	129.56	510
6600	122.10	590

Table 3: Migration Distances for Each Test Sample and Calculated Average Fragment Lengths.

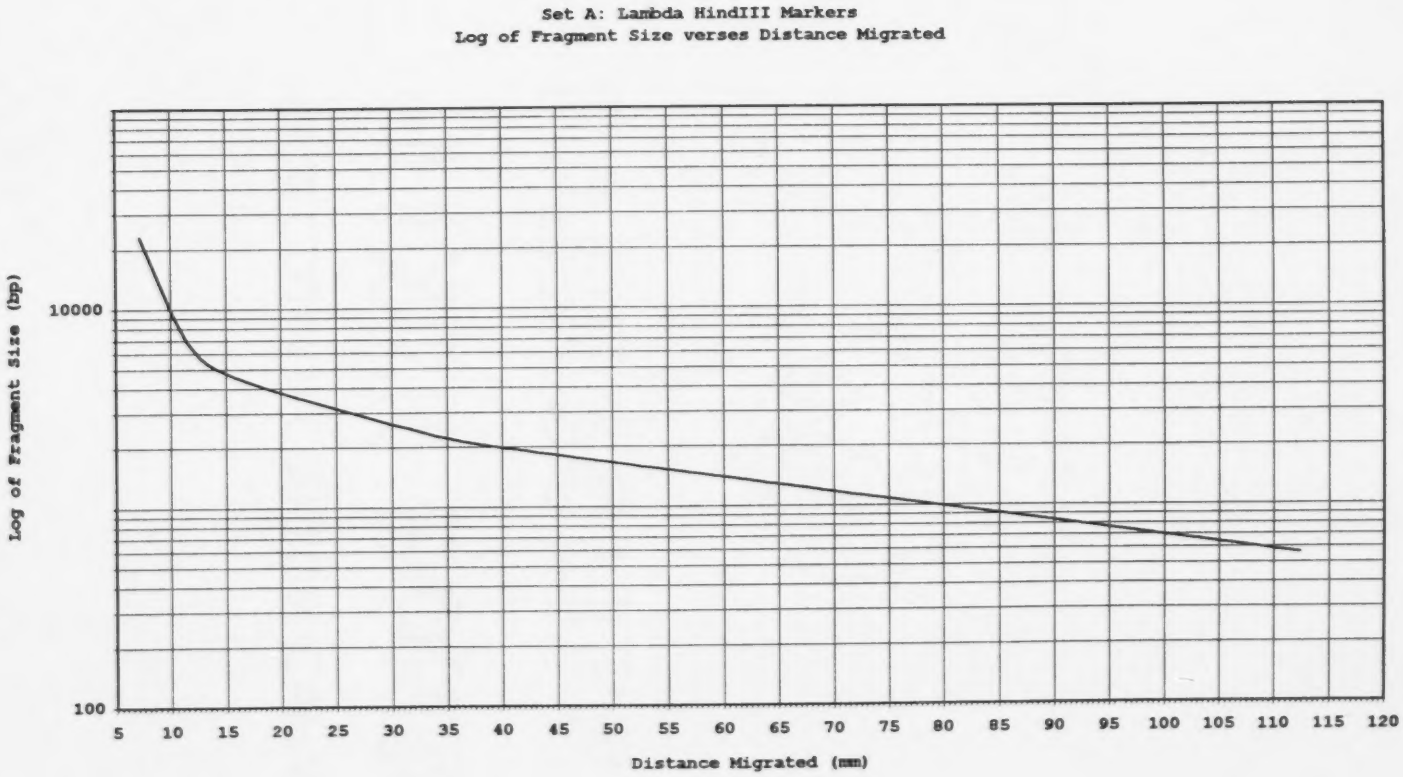
Set A: Lambda HindIII Markers
Log of Fragment Size verses Distance Migrated
Log of Fragment Size (bp)
10000
100
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120
Distance Migrated (mm)

Figure 28. Log of fragment size in base pairs verses distance migrated from well in millimetres for the Lambda HindIII markers on set A.

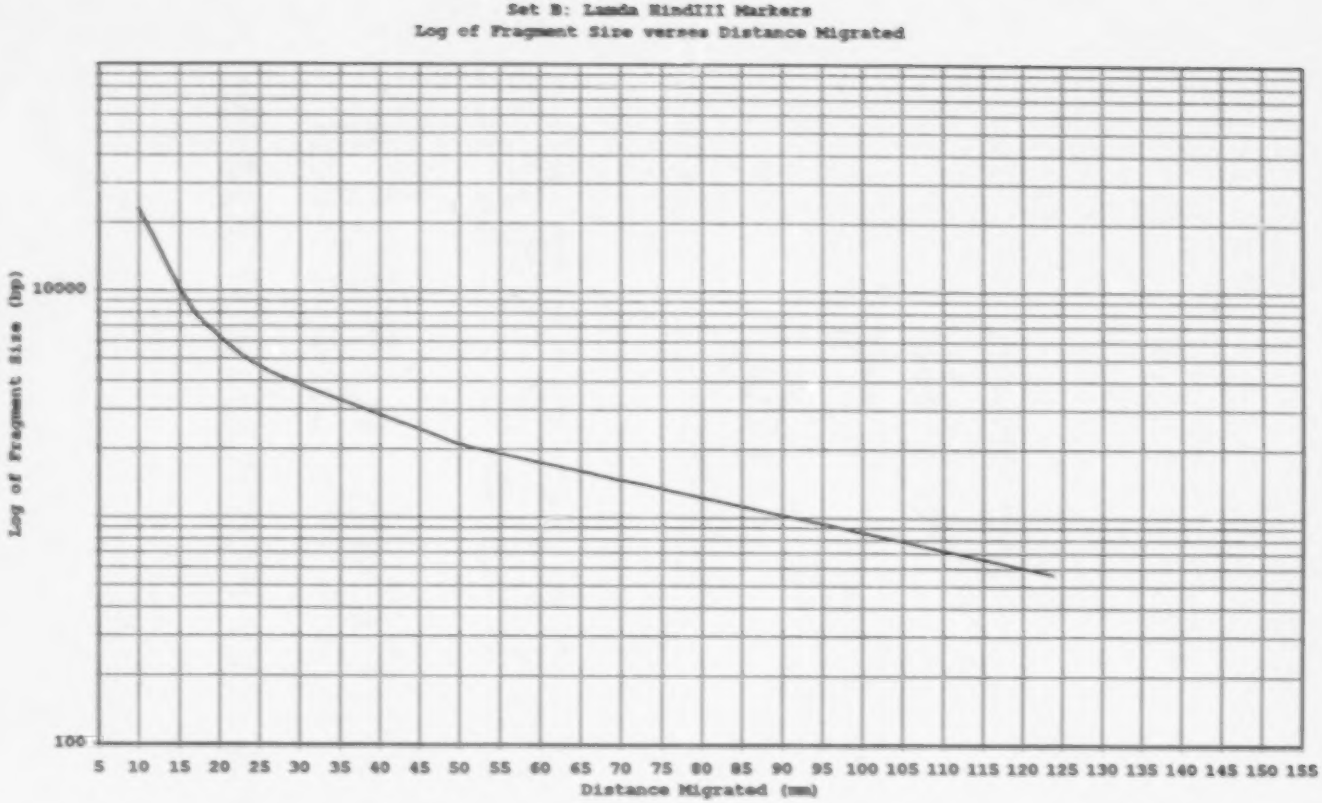
Set B: Lambda HindIII Markers
Log of Fragment Size verses Distance Migrated
Log of Fragment Size (bp)
10000
100
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120 125 130 135 140 145 150 155
Distance Migrated (mm)

Figure 29. Log of fragment size in base pairs verses distance migrated from well in millimetres for the Lambda HindIII markers on set A.

While many of the technical processes used here are well established, I am unaware of any protocols that use this specific method of analysis for determining an average fragment length value for highly degraded samples of DNA. The fabrication of this technique seemed a logical extension of existing protocols.

CHAPTER FIVE

RESULTS AND DISCUSSION: THE YIELD AND CONDITION OF DNA FROM SURFACE WEATHERED COMPACT BONE AND THEIR RELATIONSHIP TO THE TIME ELAPSED SINCE DEATH

Two complete sets of seven bovine compact bone samples aged 14 days to 18 years postmortem were independently processed and analyzed using the techniques previously outlined. DNA was successfully extracted from all of the samples. Figures 30 and 31 represent the ethidium visualized electrophoretic gels of the two sets. Set "B" was processed and extracted one month after set "A". Both sets were stored intact at room temperature until processed, and then were kept at -80 degrees Celsius once powderized. Column one and ten in each figure represents the Lambda HindIII marker used for the quality and quantity analysis of the unknown samples. Column two on each gel was not loaded with DNA and was used as a control for background fluorescence. Columns three through nine represent the samples 14 days, 6 months, 11 months, 22 months, 10 years, 12 years, and 18 years respectively.

Figure 30: Photograph of set A.

Figure 31: Photograph of Set B.

QUANTITY STUDY

The quantities of DNA extracted from each of the tested samples being is expressed in Table 4. This includes the total amount extracted from each sample, the portion of the total sample used in each electrophoretic gel, and the total quantity of DNA extracted per gram of bone processed. Figures 32 and 33 summarize this data graphically with the postmortem age being expressed in days elapsed since death, and DNA yield being expressed in micrograms of DNA extracted per gram of bone processed.

	Sample(days)	% of Total Extracted	Quan. on Gel (microgr)	Total microgr/ Sample	Microgr DNA/ gram Bone
SET A.					
	14	41.7	7.634	18.307	18.307
	180	41.7	15.758	37.789	6.298
	330	41.7	19.197	46.036	7.673
	660	41.7	9.613	23.053	3.842
	3650	41.7	4.563	10.942	1.824
	4380	41.7	1.181	2.832	0.472
	6570	41.7	1.307	3.134	0.522
SET B.					
	44	16.7	1.455	8.713	8.713
	210	16.7	6.997	41.898	6.983
	360	16.7	10.475	62.725	10.454
	690	25.0	2.638	10.552	1.759
	3680	33.3	0.998	2.997	0.499
	4410	33.3	0.577	1.733	0.289
	6600	33.3	0.598	1.796	0.299

Table 4. Summary of percent of total samples loaded on each gel, total quantity of DNA found on each gel, the total amount of DNA extracted per bone sample and the amount of DNA extracted per gram of bone processed.

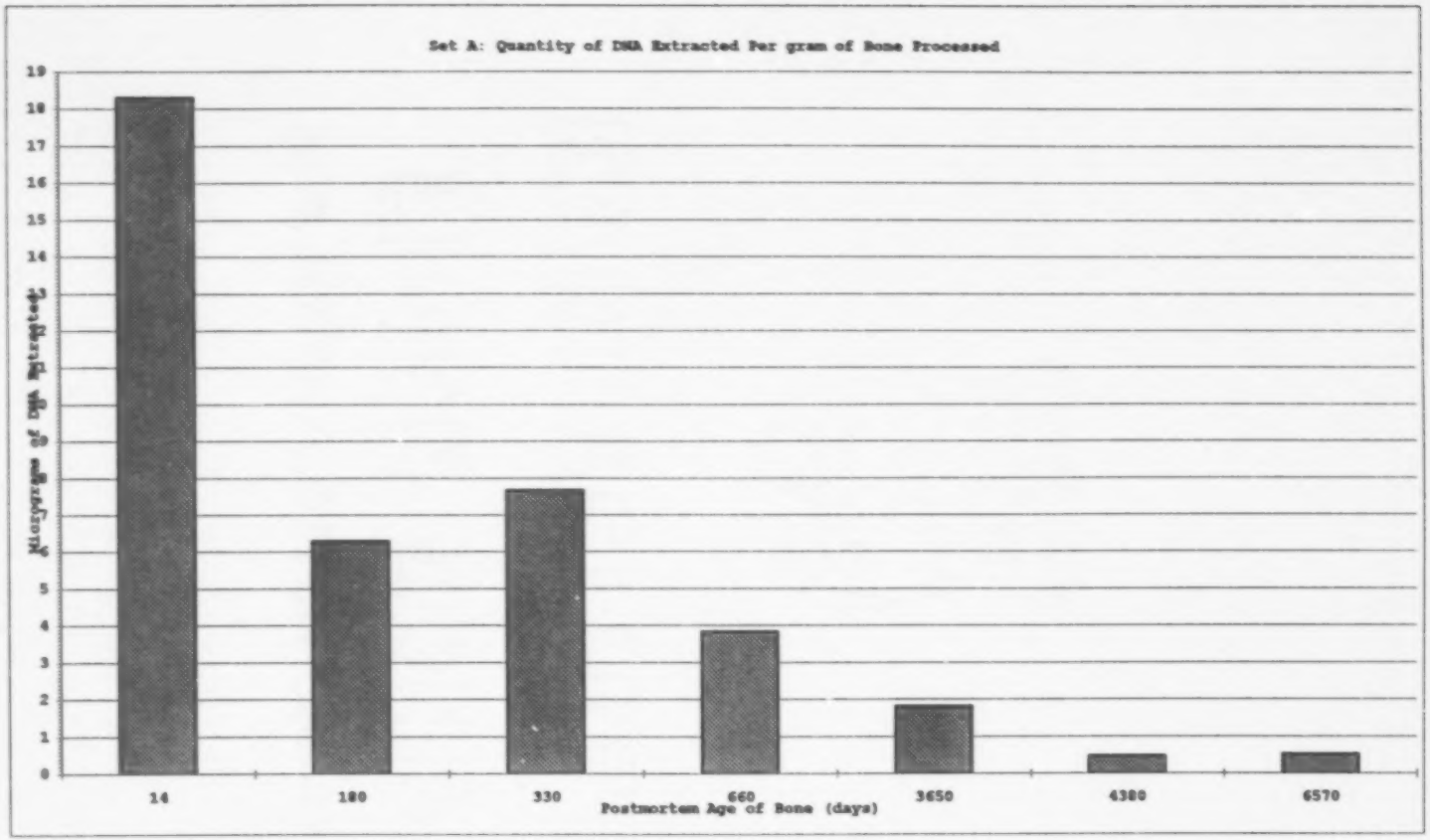

Figure 32. Quantity of DNA in micrograms extracted per gram of bovine compact bone processed. Samples are labelled by days postmortem.

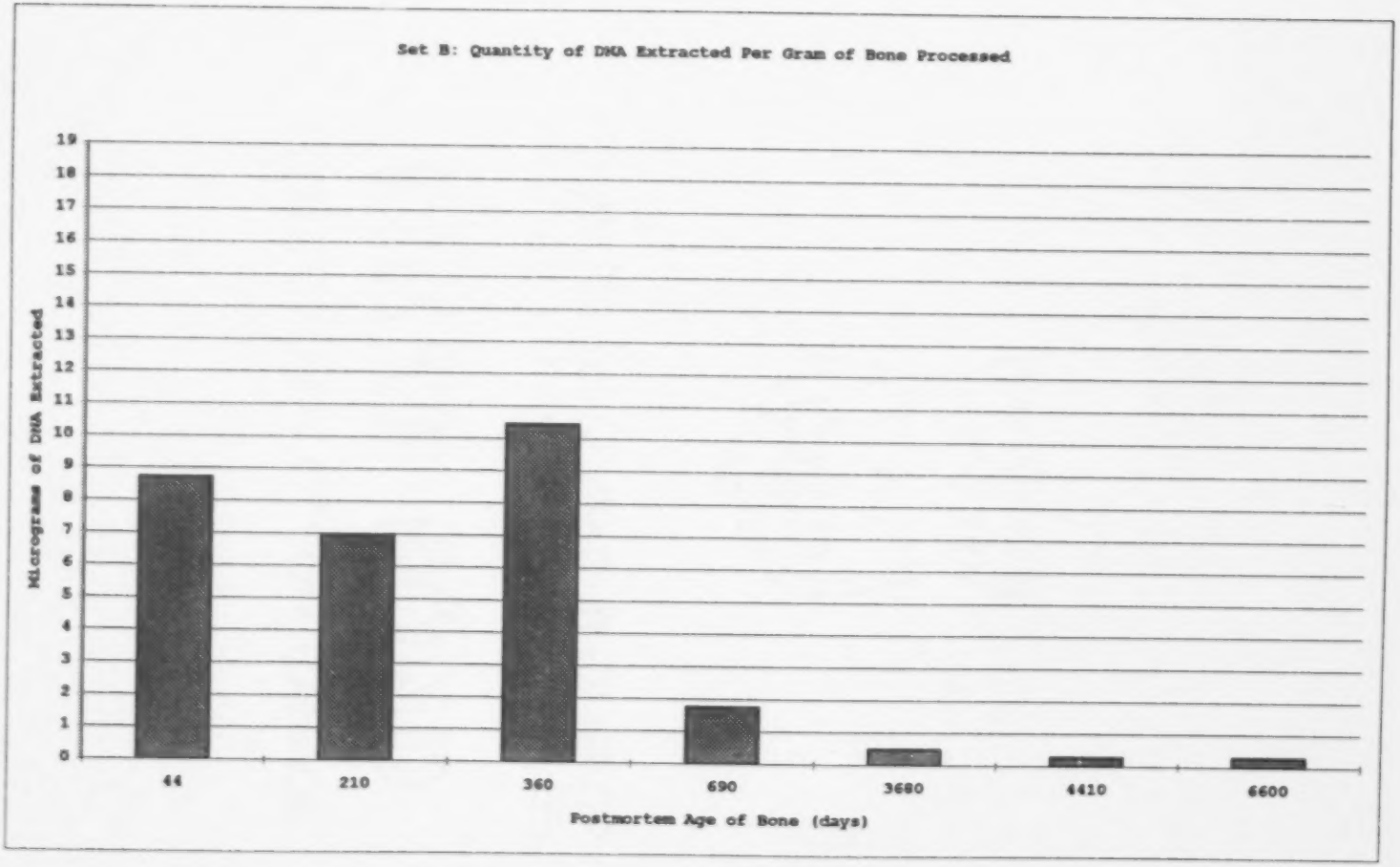

Figure 33. Quantity of DNA in micrograms extracted per gram of bovine compact bone processed. Samples are labelled by days postmortem.

As can be observed from figures 32 and 33, there is a relatively rapid decrease in the amount of DNA per gram of bone processed over the first 22 to 23 months (660/690 days) postmortem after which time the loss of DNA seems to slow considerably. In both sets of extractions, by approximately 10 years (3650/3680 days) postmortem a relatively constant quantity of DNA was extracted from each successively older sample. Although the bovine DNA hybridization experiments were not performed to document this, it seems quite logical that what is being seen is the small, but consistent, quantities of high molecular weight bacterial and/or algal DNA present in each of samples. The presence of this contamination was visually confirmed when the samples were viewed under the light microscope at high magnification. Small quantities of photosynthesizing algae could clearly be seen within the powderized bone. The high molecular weight fluorescent streaks observed in columns 7, 8 and 9 in both figures 30 and 31 are believed to represent this extraneous DNA.

The most surprising results obtained from this quantity study was the considerable amount of DNA lost between the 14 day old sample in set "A" and the same sample run 30 days later in set "B". While for each of the other samples the relative amounts of DNA extracted remained about the same between the two sets, approximately one-half the DNA was lost from this sample between 14 days and 44 days postmortem. This evidence supports the conclusion that some significant form of active DNA autodegradation is still occurring within this time period and stretches the range of active DNA degradation noted by Perry et al. (1988) far past 6 days postmortem. In light of this finding, it is obvious that our knowledge of nuclease activity after death is extremely limited and must be examined in a more rigorous manner. It is unlikely we will develop the ability to model the decompositional process without a much better

understanding of the biological factors involved.

Finally, one apparent discrepancy in the quantities of DNA extracted from the 11 and 12 month old samples can be observed. In both set "A" and set "B", significantly more DNA was extracted from the 11/12 month old samples then from the 6/7 month old samples. This fact contradicts the hypothesis that a consistent decrease in the amount of DNA extracted from successively older samples should be expected, and is thus far unexplainable unless simply credited to sample variation. Differences in the number of cells per bone area extracted, or slightly more favourable preservation conditions, could all contribute to what is seen. Only further research will help increase the understanding of the effects of sample variation on these types of systems. Until then, it is unlikely that a reliable model for using DNA quantity alone as an indicator of postmortem can be developed.

QUALITY STUDY

Table 3 (page 52) is a summary of the average fragment lengths (in base pairs) calculated for each of the seven samples listed by postmortem age of the bone (in days). Figures 34 and 35 are plots demonstrating the relationship between the condition of each sample's DNA and the time elapsed since death. Once again, set "B" was extracted and analyzed 30 days after set "A" using exactly the same protocol as previously described. It must be mentioned, however, that this data set was not calculated using bovine specific probes, and therefore, expresses the influence of extraneous microbial DNA on the results. When the ratio of bovine DNA to microbial contamination is weighted in favour

of the first, up until approximately 22 months (660 days) postmortem, the average fragment length calculated for each sample is a good reflection of the condition of the bovine DNA. For the samples older than 22 months, the ratio begins to move in favour of the microbial DNA, and thus, begins to be a better measure of the condition of the bacterial/algal DNA than of the bovine samples. Developing a better understanding of this effect may, in its self, someday be used an indicator of the time elapsed since death. By probing with a radiolabelled microbial specific, or bovine specific, probe, these ratios could be measured with a reasonably high level of accuracy. Correlating this ratio to postmortem time should produce a model for predicting the time elapsed since death.

Both graphs (Figures 34 and 35) clearly show a rapid decrease in the average fragment length of DNA over the first 660 days postmortem and then a gradual increase over the remainder of the study period. This gradual increase is a reflection of the slow loss of bovine DNA and the relatively constant level of microbial DNA present in each sample. As the low molecular weight bovine DNA is lost, the high molecular weight microbial DNA creates an artifactual increase in the overall average fragment length, even while only being present in very small quantities.

Expressed graphically, as in Figures 34 and 35, the differences in average fragment size between set "A" and set "B" are significantly enhanced. While the trend in degradation is the same in both sets, the amount of degradation that occurred between the original run (set A) and the second run 30 days later (set B) was unexpected. Although both sets of the seven samples were processes in the same manner, stored in the same manner and extracted in the same manner, a significant amount of

DNA degradation has occurred in only 30 days. This degradation is most apparent in the samples containing relatively high molecular weight DNA: The 14 day old sample primarily containing high molecular weight bovine DNA and the 10 year, 12 year and 18 year old samples primarily containing high molecular weight microbial/algal DNA. These samples exhibit active DNA autodegradation. While it seems obvious that the 14 day old sample is actively degrading due to the continuation of the autolytic process that started when the animal died, it is believed that the rapid degradation observed in the old samples is due to the death and autolysis of the indigenous microorganisms within the bone samples as a result of the storage conditions used. This explains the normalization of the curves that occurs between Figures 34 and 35 in only 30 days.

Both figures 34 and 35 show that DNA condition is closely related to the time elapsed since death. It is clear that the average fragment length of the DNA consistently decreases as the postmortem interval increases, yet without the ability to remove the affects of high molecular weight microbial DNA on the system the results of this research will remain largely preliminary. It can be stated with certainty, however, that this work in no way contradicts the original hypothesis and, while not being definitive, does support it. Ultimately, to be successful in developing a method sensitive enough for estimating the time since death, further research must include a means of controlling for microbial contamination as well as developing an understanding of how they too can be used for elapsed time determination.

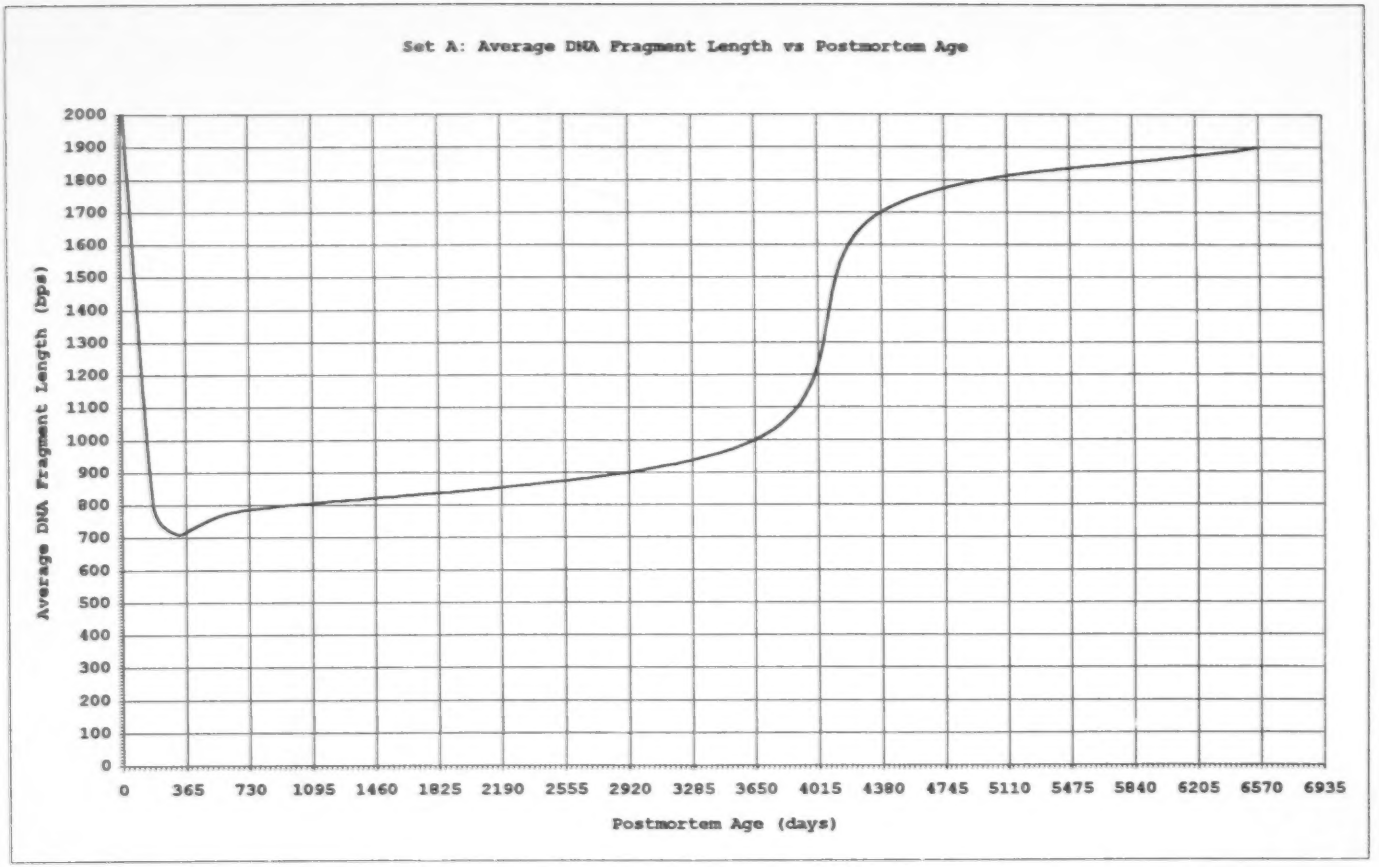

Figure 34. Plot of average DNA fragment length in base pairs verses postmortem age of bone in days for sample set A.

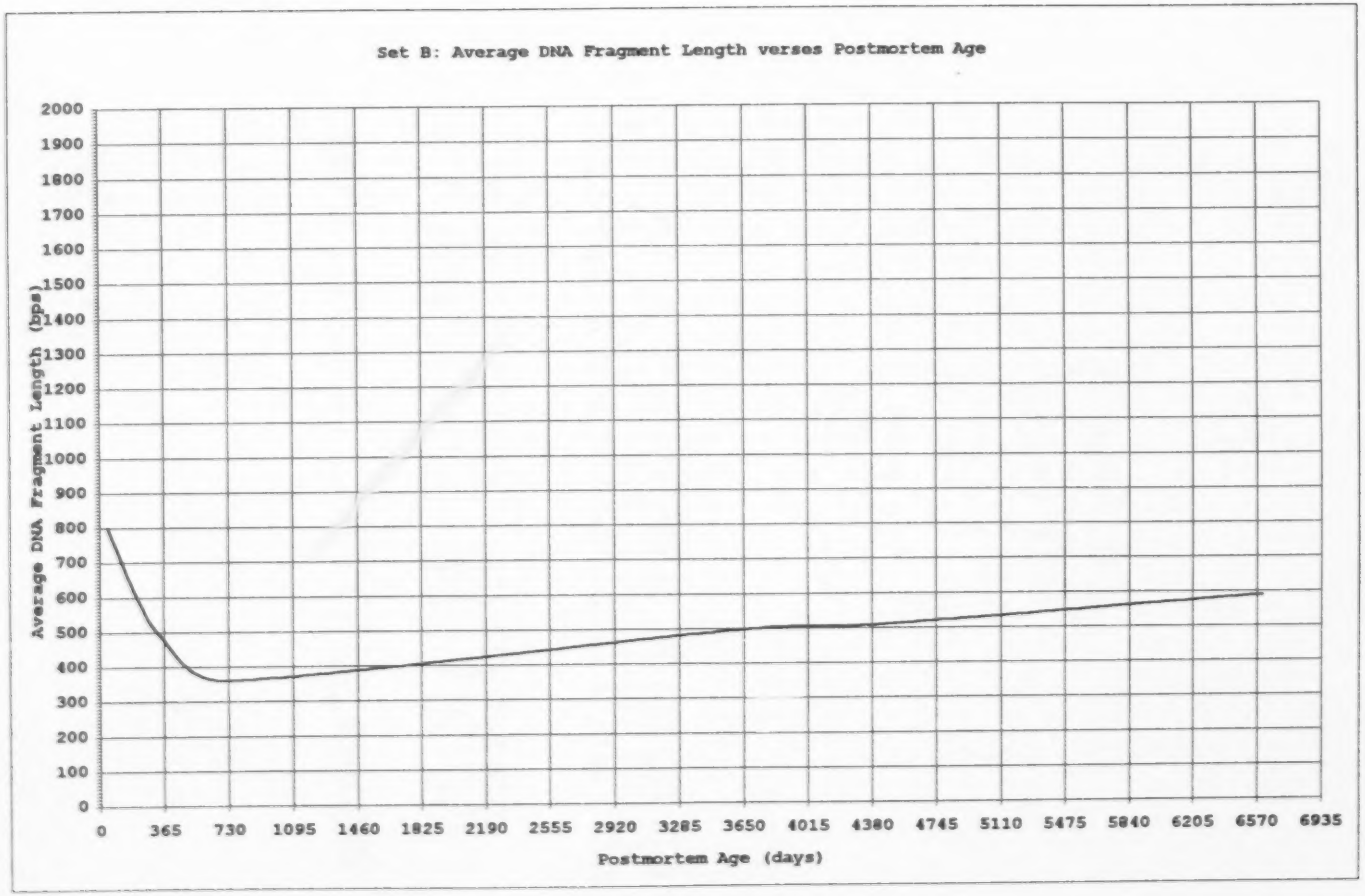

Figure 35. Plot of average DNA fragment length in base pairs verses postmortem age of bone in days for sample set A.

Although this was not unexpected, this research has shown the vast amount of quantifiable data that can result through the analysis of a bone sample. DNA was successfully extracted from samples of considerable age that had been exposed to real environmental conditions. This work has proven that the average DNA fragment length, quantity of DNA present, and nature of indigenous microorganisms can be determined for bone samples. Moreover, the high degree to which researchs can account for fluctuations in climate at a deposition site has been shown by the inclusion of 30 years of extensive climate data from only one of numerous meterological stations in Alberta (Appendix Three). While of of marginal use for this study, the need for these data for this type of research cannot be emphasized enough. For the development of a working model that can accurately estimate the time elapsed since death, it will be essential that correlations be made between samples from different regions with different climatic histories. Apprendix three outlines this variation and proves that this research is feasible.

With the application of the methods and recommendations outlined in this thesis combined with the vast amount environmental data available, we are within reach of developing a reliable system for determining time elapsed since death.

CHAPTER SIX

CONCLUSIONS

Results obtained from this research have made it possible to make a number of conclusions regarding the usefulness of this experimental system and the application of DNA quantity and quality to the estimation of the postmortem interval.

First, DNA can be successfully extracted from bone and analyzed for quantity and quality using the protocols developed for this thesis. Many of these methods are novel, and were engineered specifically to allow for the analysis of highly degraded samples from compact bone without the introduction of experimental artifacts. Further work must include a protocol for radiolabelling the samples as a means of elliminating interference by microbial DNA contamination, but this system has been shown to be effective for this unique type of analysis. This in itself is a significant achievement.

Second, the bacterial/algal DNA that was observed in each of the samples did interfere with the results from this research, however, its presence does suggest some new possibilities for developing methods for estimating the time elapsed since death. By determining the ratio of sample DNA to microbial contamination it may be possible to estimate the length of time the bone has been directly exposed to the environment. If developed this technique could be added to the growing number of tools used by forensic investigators to roughly estimate time since death.

Third, based on the evidence from the 14 day old sample and the same sample run 30 days later, the range of active DNA autodegradation determined by Perry et al. (1988), can be pushed from 6 days postmortem to a significantly longer period. Although the gap in samples makes it difficult to suggest a more precise range, active degradation is occurring between 14 days and 44 days postmortem. This fact is strong support for the need for further research on this specific topic. Our ability to predict how DNA will degrade depends on a thorough understanding of the limits of active DNA degradation.

Finally, a direct relationship between the quality and quantity of DNA and postmortem time has been shown to exist. Although the microbial DNA present in each sample makes it impossible to characterize the degradation of the bovine DNA after approximately 22 months, prior to this time a relatively rapid decrease in the quantity and quality of the bovine DNA occurs as the postmortem interval increases. At the rate of degradation seen in the samples younger than 22 months, the complete loss of DNA would be expected well before 6 years postmortem (Figures 34 and 35). Because some low molecular weight DNA can be observed in the 10, 12 and 18 year old samples, separate from the high molecular weight microbial DNA (Figures 17, 18, 19, 25, 26 and 27), this rate of degradation must slow over time. This observation supports my original hypothesis that the rate of DNA autodegradation will slow over time, becoming relatively independent of the decompositional process. Unfortunately, I was unable to characterize this phenomena in more detail in this experiment.

It is likely that, with only the results obtained here, samples could be classified as younger than 20 months, or older than 20 months in postmortem age. When applied to real forensic cases this would

contribute significantly to narrowing the number of missing persons that fit within the postmortem interval estimated, and therefore, has real and immediate practical applications in forensic identification. Within the period of May to July, 1995, in the Edmonton area alone, 3 unidentified bodies were recovered where the time elapsed since death was extremely difficult to estimate (information provided by the Office of the Chief Medical Examiner, Edmonton). The application of the most general of elapsed time determination methods, such as determining if the remains are younger than 20 months, for example, could have made a major contribution to the successful and speedy identification of these remains.

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APPENDIX ONE

SUMMARY OF BOVINE COMPACT BONE SAMPLES

Sample	Tag Number	Collection Site	Type	Individual
14 days	--	Butcher shop	Cross section of right proximal femur	Adult female
6 months	Y32	54deg25min North 110deg17min West	Medial proximal right femur	Adult female
11 months	W17	54deg25min North 110deg17min West	Medial proximal right femur	Adult female
22 months	V56	54deg25min North 110deg17min West	Medial proximal right femur	Adult female
10 years	P25	54deg25min North 110deg17min West	Medial proximal right femur	Adult female
12 years	N18	54deg25min North 110deg17min West	Medial proxiaml right femur	Adult female
18 years	H11	54deg25min North 110deg17min West	Medial proximal right femur	Adult female

Note: With exception to the 14 day old sample, the postmortem age of all the test samples were rounded to the nearest month. This was done because, as was often the case, cattle that had died were not immediately missed and the exact date of death was not known.

APPENDIX TWO

SUMMARY OF PROTOCOL

1. Remove soft tissue and bone marrow using razor blades and sand-paper. Using a Dremmel(TM) grinding tool, remove the inner and outer table of compact bone along with the fracture planes of each of the bone fragments. Surface sterilize each fragment by washing once with sterilized, deionized water and once with 98% ethanol. Ensure each fragment has completely dried before processing.

2. Freeze bone in liquid nitrogen then crush into fine bits using crushing device.

3. Transfer bits to metal blender filled with liquid nitrogen, and pulse until the sample is a fine powder. Ensure the liquid nitrogen does not completely evaporate.

4. Transfer the fine bone powder into sterile 50 ml polypropylene tubes and store at -80 degrees Celsius until prepared for DNA extraction.

5. Warm samples to room temperature and divide into 1 gram portions in separate sterile polypropylene tubes.

6. Add 2 ml of prewarmed (56 degrees Celsius) extraction buffer to each pellet.

Extraction buffer:
-0.01 molar Tris (hydroxymethyl) aminomethane (Tris)
-0.01 molar disodium EDTA-$2H_2O$
-0.1 molar sodium chloride
-0.039 molar dithiothreitol (DTT)
-100 microlitres Proteinase-K (20mg/ml)

Incubate at 56 degrees Celsius for 2 hours with intermittent shaking.

7. Allow the extraction to continue for 10 hours without agitation.

8. Add 100 ml of Proteinase-K (20mg/ml) to each tube and incubate at 56 degrees Celsius for an additional 3 hours.

9. Transfer solutions to sterile glass tubes then extract once with 0.6 volumes of phenol/chloroform/isoamyl alcohol (25:24:1). Centrifuge at 2000G for 15 minutes. Combine the aqueous phases from each tube of a given sample after the phenol extraction also in a sterile glass tube.

10. Extract solution twice with water saturated ether then remove the excess ether by warming the samples to 56 degrees Celsius while blowing a stream of nitrogen gas across the opening of the tubes.

11. Perform ethanol sedimentation by adding 2.2 volumes of ice cold 98% ethanol and 0.1 volumes of 3 molar sodium acetate and then precipitate at -20 degrees Celsius for 8 hours. Centrifuge for 15 minutes at 2000G and collect the retenate.

12. Wash the pellet 3 time with 2 ml TE buffer.

TE Buffer (pH 7.5):
-0.01 molar Tris
-0.01 molar $Na_2EDTA-2H_2O$

13. Add 100 ml of TE buffer to each pellet and store at 4 degrees Celsius until preparing for gel electrophoresis.

14. Wash samples 3 time with ice cold 70% ethanol to remove accumulated salts.

15. Transfer portion of samples and Lambda HindIII marker to sterile 2 ml epindorf tubes and add 5 ml of loading dye and enough TE buffer to increase volumes in tubes to 60 ml.

Loading dye:
-0.25% bromophenol blue
-0.25% xylene cyanol FF
-15% Ficoll (type 400; Pharmacia) in water

16. Warm tubes in heat chelator to 65 degrees Celsius to denature the DNA.

17. Immediately transfer the 60 ml samples into the loading wells on the electrophoretic gel.

2% agarose electorphoretic gel:
-Measure 3.4 g laboratory grade agarose and add 170 ml of 1 X TBE buffer.
-stir constantly while bringing to boil.
-once agarose is completely melted, take off heat and stir while solution cools to below 50 degrees Celsius.
-Add 17 ml ethidium bromide (giving a final concentration of 5 micrograms per microlitre) and stir until thoroughly mixed.
-pore solution into prepared gel plate with well former ensuring no air bubbles are present.
-let setup until completely solidified and cooled.

18. Electrophorese at 100 volts for 15 minutes until solution in wells has completely entered the gel.

19. Cover gel completely with 1 X TBE and electrophorese for 24 hours at 20 volts.

20. Transfer gel to an ultraviolet illuminator tray and photograph with a Polaroid(TM) camera using a Kodak(TM) Wratten number 22A filter and black and white film.

21. Denature the agarose gel by incubating it in 1.5 molar sodium chloride and 0.5 molar sodium hydroxide for 45 minutes in a shaker tray.

22. Rinse 3 time with sterile, deionized water.

23. Neutralize gel by incubating it in 1 molar Tris and 1.5 molar sodium chloride for 45 minutes, changing the solution after 30 minutes.

24. Transfer the DNA from the agarose gel to a solid medium using the technique of Southern (1975).

-prepare nitrocellulose membrane by cutting it to the size of the gel.
-rinse nitrocellulose paper in 2 X SSC.
-rinse gel in 10 X SSC.
-cut 2 pieces of 3MM paper; one to the size of the gel and one equalling the length of the gel but 3 times the width.
-place the larger 3MM sheet on a support draping into copious

15 X SSC and place gel on top.
-cover gel with the nitrocellulose membrane ensuring no air bubble are present between.
-place the smaller 3MM sheet on the membrane and top with approximately 10 cm of flat toweling.
-place flat glass sheet on toweling and weight with 500 g.
-cover with plastic to minimize evaporation and let transfer for 12 hours.
SSC:
-3.0 Molar Sodium Cloride
-0.3 Molar trisodium citrate (filter before use)

25. Confirm DNA transfer by viewing both the nitrocellulose membrane and the gel on the ultraviolet illuminator tray. The ethidium should be present on the nitrocellulose and absent from the gel.

26. Vacuum bake the nitrocellulose membrane at 80 degrees Celsius for 2 hours to fix the DNA to the medium.

27. Store in a dry place at room temperature until preparing for hybridization.

28. Analyze the Polaroid(TM) images, or autoradiographs, by digitalizing the images and producing density plots with the NIH gel plotting software as previously outlined. There should be no significant difference in the techniques used for analyzing autoradiographs and ethidium visualized photographs. The use of autoradiographs from bovine specific probes, however, would eliminate extraneous microbial DNA from the samples being analyzed.

APPENDIX THREE

CLIMATIC DATA FOR DEPOSITION SITE

Cold Lake, Alberta
1961 to 1992

54 degrees 25 minutes North, 110 degrees 17 minutes West
elevation 541 metres above sea level

Environment Canada, Canadian Climate Data (c) 1993

Monthly Climatic Summary For Cold Lake, Alberta (1970-1995)

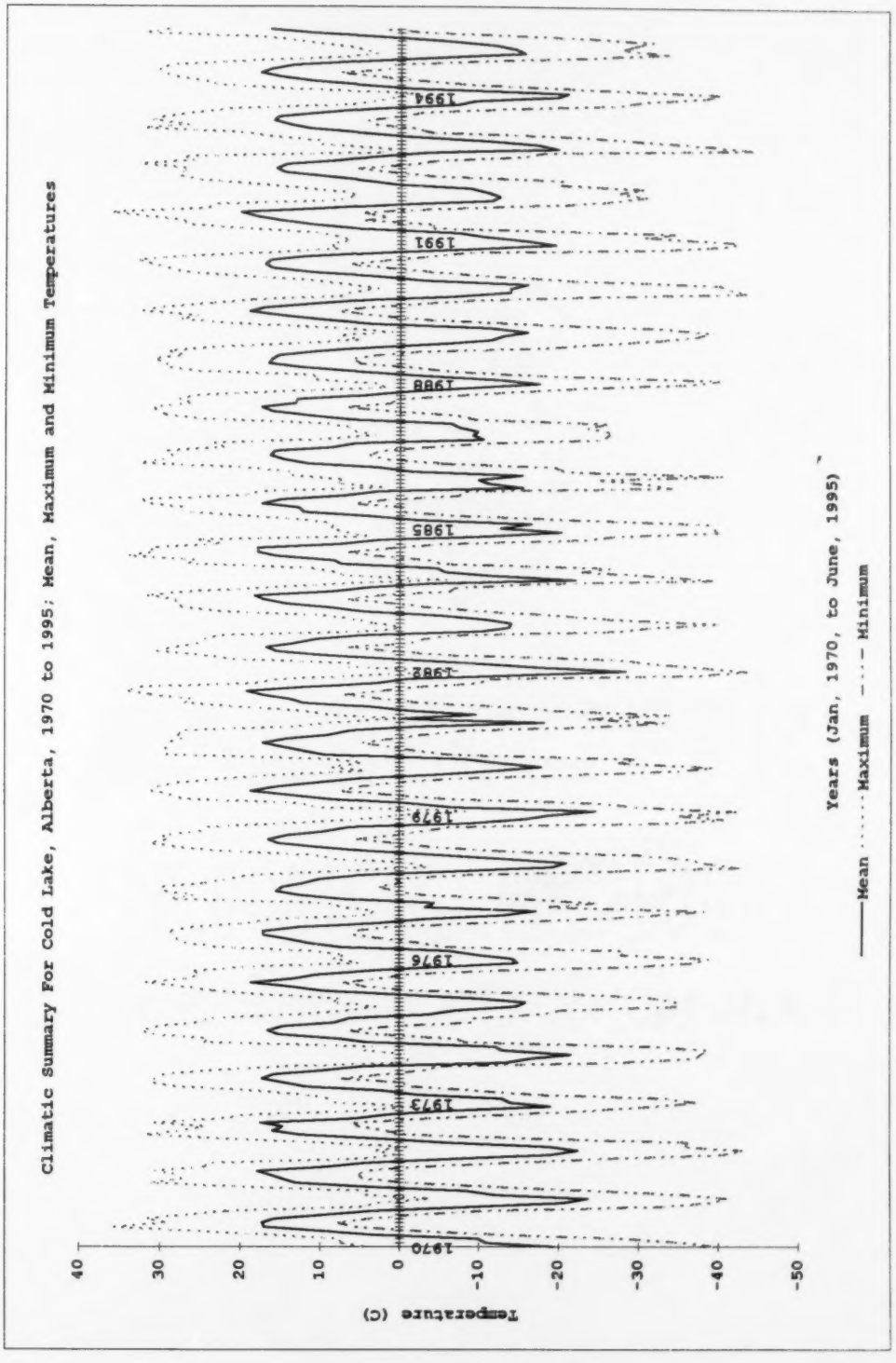
Year	Month	Mean Temp.	Max Temp.	Min Temp.	Snowfall (cm)	Rainfall (mm)	Total Prpn (mm)	Mean Hrly Rel. Humid.
1970	Jan	-19.4	4.4	-39.4	19.7	0	14.9	N
	Feb	-10.8	7.2	-36.1	19.4	0	17.3	76
	Mar	-9.8	7.8	-27.8	60.2	0	51.1	79
	Apr	3.1	16.1	-12.8	3.8	0	3.6	61
	May	10.2	26.7	-1.7	0	14.6	14.6	51
	Jun	17	35.6	5.6	0	170.6	170.6	68
	Jul	17.3	28.9	7.8	0	113.8	113.8	73
	Aug	15.9	31.7	3.9	0	64.7	64.7	72
	Sep	9.4	26.7	-2.2	5.9	32.4	38.1	73
	Oct	3.5	21.7	-10	10.7	15	25.7	74
	Nov	-9.3	4.4	-32.8	29.8	2.8	30.5	86
	Dec	-20.5	2.8	-38.9	34	0	34	N
1971	Jan	-23.7	-3.9	-41.1	27.9	0.3	27.4	N
	Feb	-12	4.4	-36.1	8.5	0	8	80
	Mar	-8.3	3.9	-23.9	13.6	0	13.4	77
	Apr	3.6	19.4	-12.2	8.4	0	7.3	61
	May	13.1	31.1	-1.1	0	48.8	48.8	46
	Jun	14.6	27.2	4.4	0	67.7	67.7	71
	Jul	16.3	27.8	5	0	179.3	179.3	74
	Aug	17.8	30.6	4.4	0	55.9	55.9	72
	Sep	9.2	24.4	-2.8	2.5	21	23.5	74
	Oct	4.3	23.9	-13.3	5.6	1.5	7.1	66
	Nov	-7.1	4.4	-26.1	21.9	3.6	21.9	82
	Dec	-18.8	0.6	-40.6	40.6	0	38.8	N
1972	Jan	-22.4	2.8	-43.3	29	0	28.3	N
	Feb	-18.9	-1.1	-35.6	22.3	0	19.3	79
	Mar	-5.8	10	-36.7	32.6	3.8	31.7	72
	Apr	2.2	21.7	-17.2	8.2	9	16.7	59
	May	12.4	31.7	-1.1	0	19.8	19.8	52
	Jun	15.9	27.2	1.7	0	42.2	42.2	59
	Jul	14.6	24.4	5	0	94.9	94.9	71
	Aug	17.4	30.6	5.6	0	49.6	62	69
	Sep	5.5	22.2	-3.9	15	33.2	47.5	72
	Oct	1.5	18.9	-13.9	1.9	10.2	12	63
	Nov	-5.7	4.4	-23.9	28.9	1.1	29.5	86
	Dec	-19	-1.7	-32.2	14	0	13.5	71
1973	Jan	-13.9	7.8	-37.2	21.3	0	16.8	74
	Feb	-12.9	8.9	-33.3	16.6	0	15	67
	Mar	-2.3	11.7	-16.7	9.9	0	7.9	68
	Apr	2.5	18.9	-9.4	26.4	10.2	36.6	58
	May	11.7	27.8	-1.7	0	35.1	35.1	61
	Jun	14.6	31.1	0	0	86.7	86.7	71
	Jul	17.2	30.6	7.2	0	75.3	75.3	68
	Aug	15.8	29.4	1.7	0	100.4	100.4	72
	Sep	9.9	23.9	-3.3	2.5	79.9	82.4	69
	Oct	4.4	13.9	-5	6.4	42.2	49.5	72
	Nov	-13.4	-1.1	-27.8	64.4	0.3	51.6	76
	Dec	-16.5	-1.1	-37.2	17.9	0	15.8	73
1974	Jan	-21.6	-1.1	-37.8	45.9	0.3	36.4	63
	Feb	-12.8	4.4	-38.9	22.7	0	20.1	70
	Mar	-12.1	3.3	-31.1	42.9	0	31.5	68
	Apr	4.1	24.4	-8.3	5.4	19	21.8	64
	May	7.6	23.9	-7.2	29.5	41.5	70.7	66
	Jun	15.2	31.1	3.3	0	108.5	108.5	64
	Jul	16.5	32.2	6.1	0	114.4	114.4	73
	Aug	14.6	30	0.6	0	53.9	53.9	75
	Sep	8.2	25	-9.4	15.7	59	74.2	72
	Oct	6.3	23.9	-9.4	0	19.2	19.2	63
	Nov	-3.7	12.8	-17.8	20.7	0	19.3	77
	Dec	-7.1	6.1	-20.6	4.4	0.3	4	80
1975	Jan	-14.6	2.8	-35	32.1	0	29.1	74
	Feb	-15.9	8.3	-35	22.8	0	20.6	63
	Mar	-10	7.2	-32.2	33	0	25.1	68
	Apr	1.1	17.8	-22.8	18.7	13	32.5	67
	May	9.7	23.3	-1.7	0	45.5	58.2	62
	Jun	13.8	27.2	4.4	0	121.8	121.8	72
	Jul	18.6	31.7	7.2	0	61	61	70
	Aug	13.1	26.1	2.2	0	83.3	83.3	77
	Sep	10.5	25	-1.1	0	11.6	11.6	72
	Oct	4.1	25.6	-11.1	12	3.8	16.3	73
	Nov	-5.4	18.9	-29.4	9.7	0	7.7	71
	Dec	-14.9	5	-36.7	38.7	1.5	34.8	72
1976	Jan	-14.2	8.3	-38.9	13.4	7.7	19	69

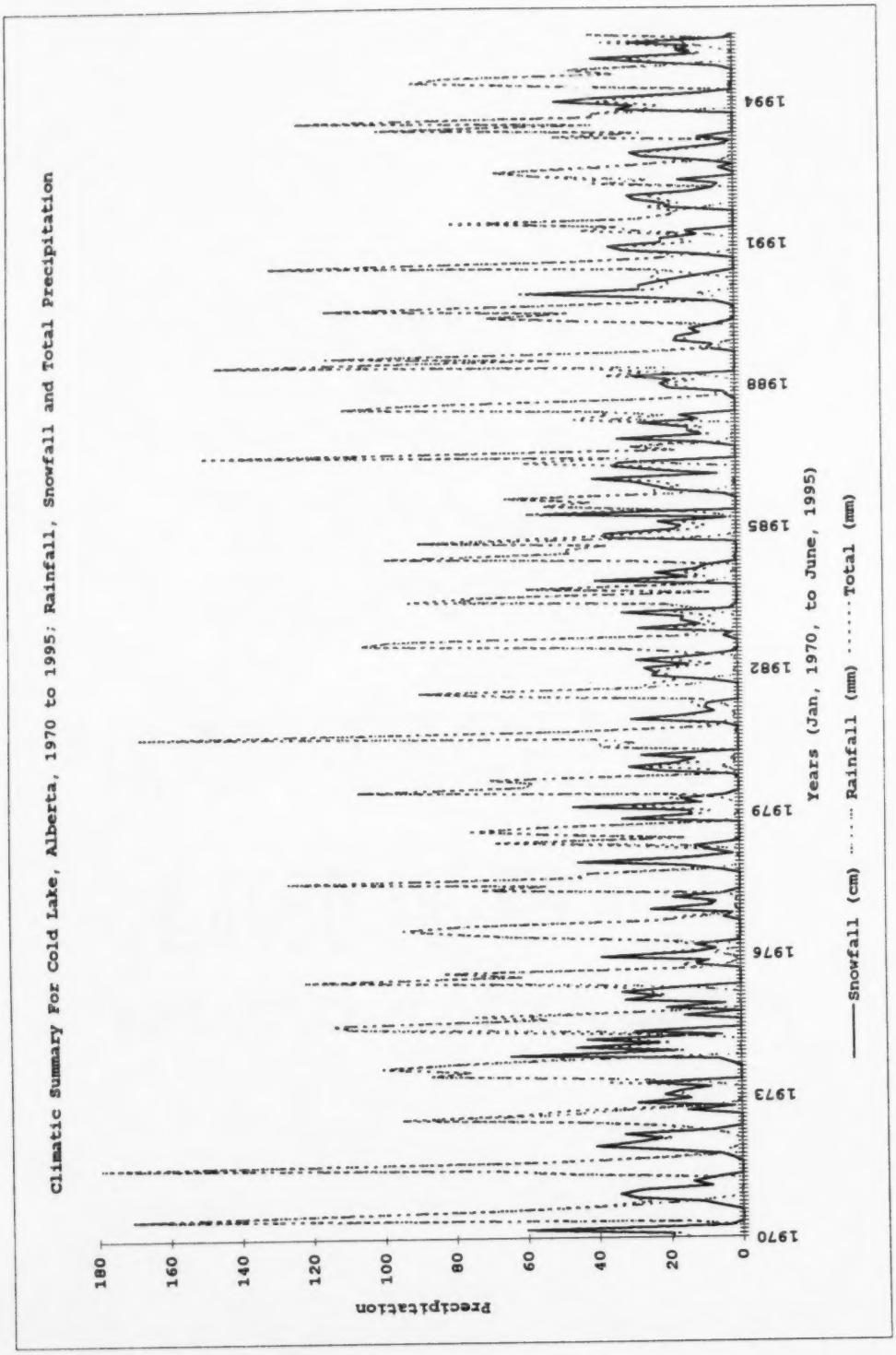
	Feb	-9.9	6.7	-27.8	6.7	0.5	6.2	69
	Mar	-6.2	11.1	-28.3	12.6	0	12.6	66
	Apr	7.2	22.8	-5.6	1	21.4	22.4	59
	May	11.5	26.7	-2.8	0	61	61	53
	Jun	13.9	27.8	1.7	0	78.9	78.9	67
	Jul	17	27.8	5.6	0	94.3	94.3	71
	Aug	17.1	28.9	5.6	0	77.2	77.2	74
	Sep	12.5	25.6	1.1	0	22.9	22.9	69
	Oct	2.6	18.9	-9.4	4.6	11.4	15.8	68
	Nov	-3.5	15.6	-24.4	2.1	0.5	2.3	63
	Dec	-13.1	3.9	-32.2	24.8	0.3	25.2	74
1977	Jan	-17.2	3.1	-38.4	8.2	1.6	9.1	65
	Feb	-3.3	6.9	-18.4	6.8	1.6	7.8	72
	Mar	-4.4	9	-24.9	18.6	0.5	18.9	71
	Apr	6.6	28.6	-15.4	4.9	4.9	9.8	52
	May	12.5	25.7	-3	0	72.3	72.3	64
	Jun	15.4	29.6	0.7	0	53.9	53.9	61
	Jul	14.7	29.6	2.5	0	126.4	126.4	75
	Aug	12.1	24.4	-0.5	0	43.1	45.7	73
	Sep	10	21.3	0.4	0	42.4	42.4	78
	Oct	5.1	18.7	-7.8	5.3	7.3	12.6	63
	Nov	-8	7.2	-27.6	15.7	0	14.2	73
	Dec	-19.1	-3.6	-42.8	45	0	37.3	N
1978	Jan	-21	-1.1	-38	13.5	0	11.6	65
	Feb	-13.7	7.7	-36.7	1.8	0.7	2.7	N
	Mar	-4.9	10.7	-29.5	8.4	0	8.2	63
	Apr	3.6	21.9	-13.1	12.4	16.3	28.7	64
	May	10.2	28	-4	1.7	65.7	68.6	67
	Jun	15.2	31.1	0.5	0	14.8	15.4	66
	Jul	16.4	28.8	5.3	0	48.4	48.4	65
	Aug	14.1	29.5	0.3	0	75.1	75.1	73
	Sep	9.5	24.9	-2.9	0	54.2	54.2	79
	Oct	6.4	23.9	-6.5	0	4.7	4.7	62
	Nov	-8.8	14.3	-32.7	32.5	1.4	28.1	71
	Dec	-15.7	4.2	-40.8	13	1	10.8	73
1979	Jan	-18.8	0	-34.8	14	0	7.9	74
	Feb	-24.6	-9	-42.3	46.2	0	30.2	N
	Mar	-6.6	10.4	-30.2	11.5	0.6	9.8	70
	Apr	-0.6	12.7	-23.8	16.1	4.7	19.2	68
	May	8.4	26.9	-6.4	3.8	11.8	14.4	56
	Jun	13.9	28.6	1.3	0	106.9	106.9	71
	Jul	18.6	31	7.1	0	59.8	59.8	74
	Aug	15.4	30.1	5	0	57.2	57.2	79
	Sep	11.2	27.8	0.8	0	69.4	69.4	79
	Oct	5.7	22	-6.6	1.5	5.6	7.1	76
	Nov	-4	15.4	-21	22	0	6.5	78
	Dec	-12	4.2	-34.8	30.5	5.3	26.9	84
1980	Jan	-17.8	8.4	-39.4	17.4	0	11.3	76
	Feb	-12.2	4.5	-27.2	12.5	2	10.8	76
	Mar	-8.1	8.3	-29.8	27	0	24.1	73
	Apr	9.3	29.4	-6.3	0	1.6	1.6	49
	May	12	29.2	-2	0	37.5	37.5	53
	Jun	14.8	29.2	1.5	0	28.6	38.9	66
	Jul	17.1	29	4.6	0	39.8	39.8	66
	Aug	14.1	27	0.5	0	157.6	168	80
	Sep	9	27.7	-3	0.8	46.9	47.7	80
	Oct	6.2	27.4	-8.3	0	6.8	6.8	69
	Nov	-2	13.5	-24.4	4.4	3.8	10.1	77
	Dec	-18.1	8.8	-33.3	29.7	2.8	27.3	71
1981	Jan	N	N	-23.6	15.1	0	12.7	85
	Feb	-9.6	8.7	-33.9	6.7	2	6.2	72
	Mar	-1.3	12.2	-19.7	9.4	1.8	9.8	65
	Apr	4	20.5	-13.1	8.7	5.5	13	56
	May	12.1	26.9	-2.7	0	10.2	10.2	48
	Jun	13.7	25.9	1.9	0	63.1	56.1	62
	Jul	17.3	31.1	6.8	0	89.1	89.1	70
	Aug	19.1	34.1	2.2	0	27.8	27.8	63
	Sep	11.8	32.1	-2.4	0	10	24	67
	Oct	2.8	18	-10	7.3	11	17.3	74
	Nov	-1.1	14.7	-11.3	23.8	1	18	80
	Dec	-14.6	3.6	-37.3	23	1.6	23.4	N
1982	Jan	-28.4	-7.6	-43.7	26	0	17.2	N
	Feb	-17.4	6.3	-38.2	13.7	0	6.9	65
	Mar	-9	5.8	-31.3	28.2	0	18.3	68
	Apr	0.3	20.6	-27	11.5	1	12.3	59
	May	9.6	24.6	-6	1.6	15.8	16.9	59

Jun	15.4	30.3	0.8	0	35.8	35.8	59
Jul	16.7	28.1	6.3	0	104.9	105.1	75
Aug	13.3	25	-1.3	0	97	97	76
Sep	10.6	24.4	-3.1	3.8	11.4	15.2	68
Oct	5.1	22.2	-6.6	1.4	10.2	11.6	69
Nov	-10.3	3.3	-27.5	27.6	1	17.5	76
Dec	-13.7	-0.6	-31.4	10.3	0	5.8	81
1983 Jan	-14	3.2	-39.9	15.7	0	9.9	75
Feb	-10.9	5.5	-26.9	14.9	0	7.2	71
Mar	-5	9.4	-20	32.1	5.2	17.5	69
Apr	4.4	22.3	-11	2	10.2	12.2	56
May	8.[illegible]	27	-4.3	0.6	19.5	20.1	60
Jun	14.2	28	0.5	0	78.4	92	70
Jul	17.1	28	6.4	0	72.5	72.5	77
Aug	18.1	31.8	2.7	0	7.5	7.5	66
Sep	8.6	25.9	-6.3	0	58.8	58.8	72
Oct	4.3	16.3	-7	0	12	12	69
Nov	-3.9	7.6	-18.6	39.4	5.3	31.5	86
Dec	-22.1	-6	-39.5	13.8	0	8.7	N
1984 Jan	-11.5	6.8	-31.9	22.6	1.6	15.3	N
Feb	-5.6	8.1	-22.8	11.3	0	8.1	69
Mar	-4.7	10.9	-26.5	8.8	0	6.4	64
Apr	7.4	26.4	-5.2	0	28.9	28.9	51
May	8.3	25.5	-3.9	0.6	97.9	98.3	58
Jun	15.5	33.9	3	0	46.6	46.6	60
Jul	17.7	30.5	6.4	0	47.4	47.4	63
Aug	17.7	31.2	0.6	0	36.2	36.2	65
Sep	7.7	22.3	-5.2	0.4	88.8	89.2	71
Oct	2.5	25.5	-23.5	36.6	6.6	48.4	71
Nov	-11	3.7	-27.2	36.8	0	26.3	N
Dec	-20.3	7.2	-40	17.5	0	11.9	72
1985 Jan	-12.7	8.6	-39.5	15.6	0	8.6	74
Feb	-16.5	8.4	-39	21.7	0	15.6	71
Mar	-2.3	11.5	-23	2.6	0	1.2	67
Apr	4.3	19.7	-7.5	53.8	7.4	58.8	60
May	12.1	27.2	-0.4	0	21.6	21.6	55
Jun	12.6	26.8	-0.7	0	53.5	53.5	63
Jul	17.2	31.3	5.7	0	40.6	40.6	64
Aug	14.8	32.1	1.3	0	64.6	64.6	65
Sep	7.2	19.7	-7.4	3.2	18	21.2	70
Oct	2.7	17.2	-7.7	17.9	4.4	22.7	69
Nov	-15.5	1.8	-34.2	22.4	0	15.6	72
Dec	-11	7.9	-30.5	28	0.8	21.2	72
1986 Jan	-9.9	6.9	-25.1	40	0.2	35.7	71
Feb	-15.4	14.1	-40.8	5.6	0	3.9	63
Mar	-1.5	13.9	-20.8	20.9	0.3	19	69
Apr	2.9	20.1	-19.6	34.2	0.8	27.3	55
May	11.6	32.5	-5.6	32.7	26.4	59.7	60
Jun	14.7	29.5	1.6	0	30.8	35	61
Jul	16.2	28.4	3.8	0	142.7	150.1	76
Aug	15.7	29.2	2.5	0	16.8	16.8	66
Sep	8.5	22	-3	0	29.3	29.3	73
Oct	5.4	23.2	-10.5	9.3	4.2	12.7	68
Nov	-10.5	9.8	-25.5	33	1.2	28.6	74
Dec	-9.2	3	-26.2	9.6	1	7.2	75
1987 Jan	-9.8	5.6	-26.3	13.4	0	12.6	71
Feb	-7.5	5.8	-23.6	13.5	0	9	75
Mar	-6.4	9.7	-26.4	26.4	0	23.8	76
Apr	6.2	23.8	-9.6	10.4	34.4	45	62
May	11.8	29.3	-7.2	15.6	19.7	35.3	56
Jun	16	30.3	0.6	0	37.9	37.9	60
Jul	17.3	30.8	6.4	0	110.3	110.3	70
Aug	13.1	25.8	1.2	0	91.1	91.1	N
Sep	12.8	27.6	0	0	23.6	23.6	70
Oct	4.6	25.2	-7	2.2	3.4	5.6	63
Nov	-1.4	13.3	-15.4	3.4	0.4	5.2	76
Dec	-8.9	1.7	-23.4	18.5	0	13.2	86
1988 Jan	-17.6	3	-37	20.8	0	18.8	73
Feb	-12.6	11	-40.4	17.9	0	11.1	N
Mar	-1.9	10.7	-17.3	28.9	7.8	36.2	77
Apr	5.2	25	-6.8	0.4	22.1	23.9	53
May	12.4	29.4	-1.6	0	19.4	41.4	59
Jun	16.4	29.1	4.9	0	146	146	74
Jul	16	30.7	6	0	51.7	51.7	76
Aug	15.2	27	3.5	0	114.7	114.7	78
Sep	10.3	28.9	-3	0	27.3	26.9	78

	Oct	4	23.5	-17.9	7.8	2.6	9.4	77
	Nov	-5.4	12.5	-25.4	6.2	5	9.5	91
	Dec	-11.4	5	-32.5	16.8	0	13.3	83
1989	Jan	-13	7.5	-37.5	16.1	0.4	14	78
	Feb	-16.1	0.9	-39	10	0	9.6	74
	Mar	-11.3	8.9	-33.1	12.4	0	10.2	75
	Apr	3.4	21	-16.5	6.6	3.2	9.4	61
	May	10	27.5	-3.2	1.2	43.9	45.7	62
	Jun	14.6	25.3	2.4	0	69	69	73
	Jul	18.8	32.2	7.5	0	46.6	47.2	72
	Aug	17.1	30	4.9	0	115.1	115.1	77
	Sep	10.6	23.5	-2.9	1.2	48.9	50.1	80
	Oct	4.2	18.3	-9.8	5.6	3.8	9.4	77
	Nov	-6.7	7.4	-23.9	25.3	4.8	24.7	87
	Dec	-13.9	5.9	-43.7	56.2	10	60	N
1990	Jan	-13.9	2.7	-41.6	26.7	0.6	22	85
	Feb	-16.1	7.1	-40.3	26.6	0.6	18.2	79
	Mar	-2.9	11.1	-22.8	21.3	0	18.6	77
	Apr	3	20.6	-11.7	14.2	2.4	23	70
	May	10.9	27.1	-5	7	11.6	20.6	61
	Jun	16.1	29.1	1.2	0	22	22	63
	Jul	16.9	31	6.1	0	130.6	130.6	N
	Aug	16.3	32.7	1.6	0	48.9	48.9	75
	Sep	11.9	27	-3.5	0	21	21	73
	Oct	1.7	15.4	-8.5	11.2	6.6	17.8	78
	Nov	-9.4	7.6	-33.1	30.8	0	30.2	79
	Dec	-19.6	8.7	-41.7	35.1	0	27.7	77
1991	Jan	-16.2	6.2	-42.6	20.4	0	11.6	N
	Feb	-10.5	7.7	-30.8	20.4	0.2	15.8	N
	Mar	-7.3	10.5	-34.9	10.6	7	15.6	N
	Apr	5.8	20.2	-4.3	13.4	29	42.8	N
	May	11	26.6	-4.1	0	32.2	32.2	N
	Jun	15.1	27.2	4.4	0	72.2	80.2	N
	Jul	17.9	31.6	1.5	0	27	27	N
	Aug	19.9	36.1	4.9	0	17.8	17.8	N
	Sep	10.2	24.2	-5.3	0	16.4	16.4	N
	Oct	-0.1	23.2	-20.4	18.8	7.6	23.6	N
	Nov	-11.5	8.3	-26.9	19.4	0	15.3	N
	Dec	-12.5	5.4	-31.1	27.9	0	20.5	N
1992	Jan	-11	6.5	-27.5	29.6	0	24.2	N
	Feb	-8.8	12.4	-30.7	12.2	0	11.1	N
	Mar	0.5	17.9	-20.1	6	0	5.6	N
	Apr	4.6	24.6	-20.6	4.4	35.8	39.8	N
	May	9.2	27.7	-4.8	15.4	24.3	38.1	N
	Jun	14.6	27.3	1.1	0	56.6	56.6	N
	Jul	15.5	26.5	5.3	0	67	67	N
	Aug	14.2	32.4	-1.5	4	38.4	44.4	N
	Sep	7.8	25.7	-4.8	0	24.1	24.1	N
	Oct	3.5	27.3	-15.4	9.1	2.9	12.1	N
	Nov	-3.5	2.6	-18.3	25.8	0.2	23.8	N
	Dec	-19.9	0.7	-44.4	28.8	0	24.6	N
1993	Jan	-17.3	10.2	-38.8	14.9	0	11.3	N
	Feb	-10.8	10	-31.2	3.6	0	2.7	N
	Mar	-0.6	16.3	-22.6	0.8	0	0.5	N
	Apr	4.9	19.3	-4	9.8	43.2	50.6	N
	May	11.4	32.1	-1.9	0	26	26	N
	Jun	13.8	26.5	3	0	99.9	99.9	N
	Jul	15.8	31	4.1	0	39.6	39.6	N
	Aug	15.3	29.4	0.4	0	123	123	N
	Sep	9.5	25.1	-3.1	0	39.7	39.7	N
	Oct	3.6	21.5	-13	0.2	39.2	39.2	N
	Nov	-7.1	6.1	-27.9	32.2	0	24.3	N
	Dec	-9.4	3.4	-30.6	27.9	0	21.1	N
1994	Jan	-19.8	0.2	-38.7	49.9	0	37.7	N
	Feb	-21.1	-1.6	-40.2	38	0	28.7	N
	Mar	-1.7	14.3	-23.6	11.4	0	8.6	N
	Apr	4.5	22.7	-10	0	2.4	2.4	N
	May	11.1	24.6	-2.6	1	54.5	55.8	N
	Jun	14.8	28.3	2.1	0	90.2	90.2	N
	Jul	17.5	29.6	7.6	0	80.7	80.7	N

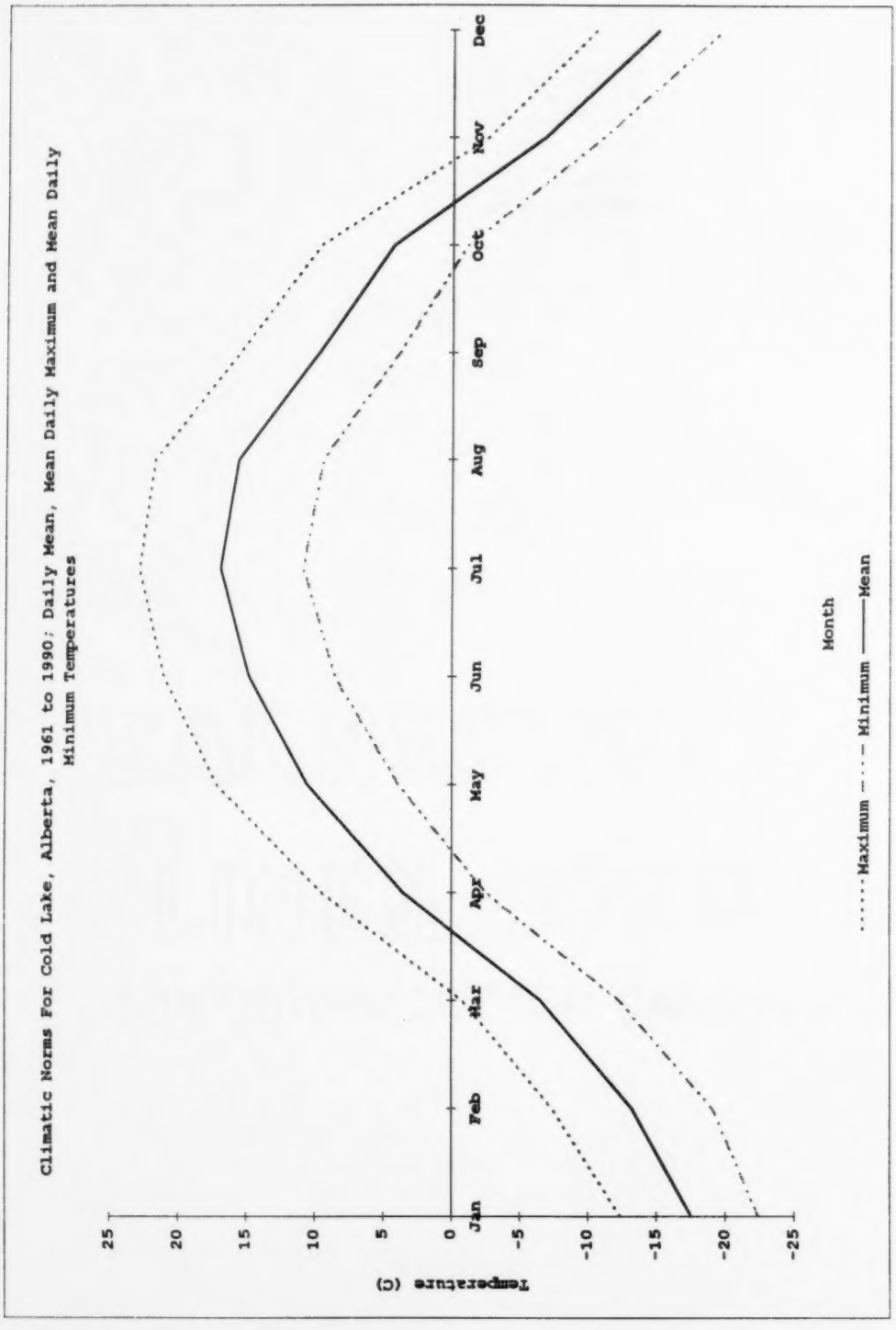
Aug	16.2	30.2	1.5	0	33.3	33.3	N
Sep	13.2	28.5	1.1	0	45.7	45.7	N
Oct	4.6	19.8	-10	4.6	21	24.5	N
Nov	-6.6	6.5	-24.4	25.4	2	21.2	N
Dec	-15.7	2.8	-34.1	39.4	0	29.8	N
1995 Jan	-14.5	4.8	-28	11.3	0	8.5	N
Feb	-12	8	-29.5	15.5	1	12.4	N
Mar	-5.7	12.6	-31.9	12	9.2	18.3	N
Apr	1.6	16.4	-21.6	29.2	14.6	36.7	N
May	10.6	32	-4.4	2.4	6.2	8	N
Jun	16.2	28.8	2.1	0	40.3	40.3	N

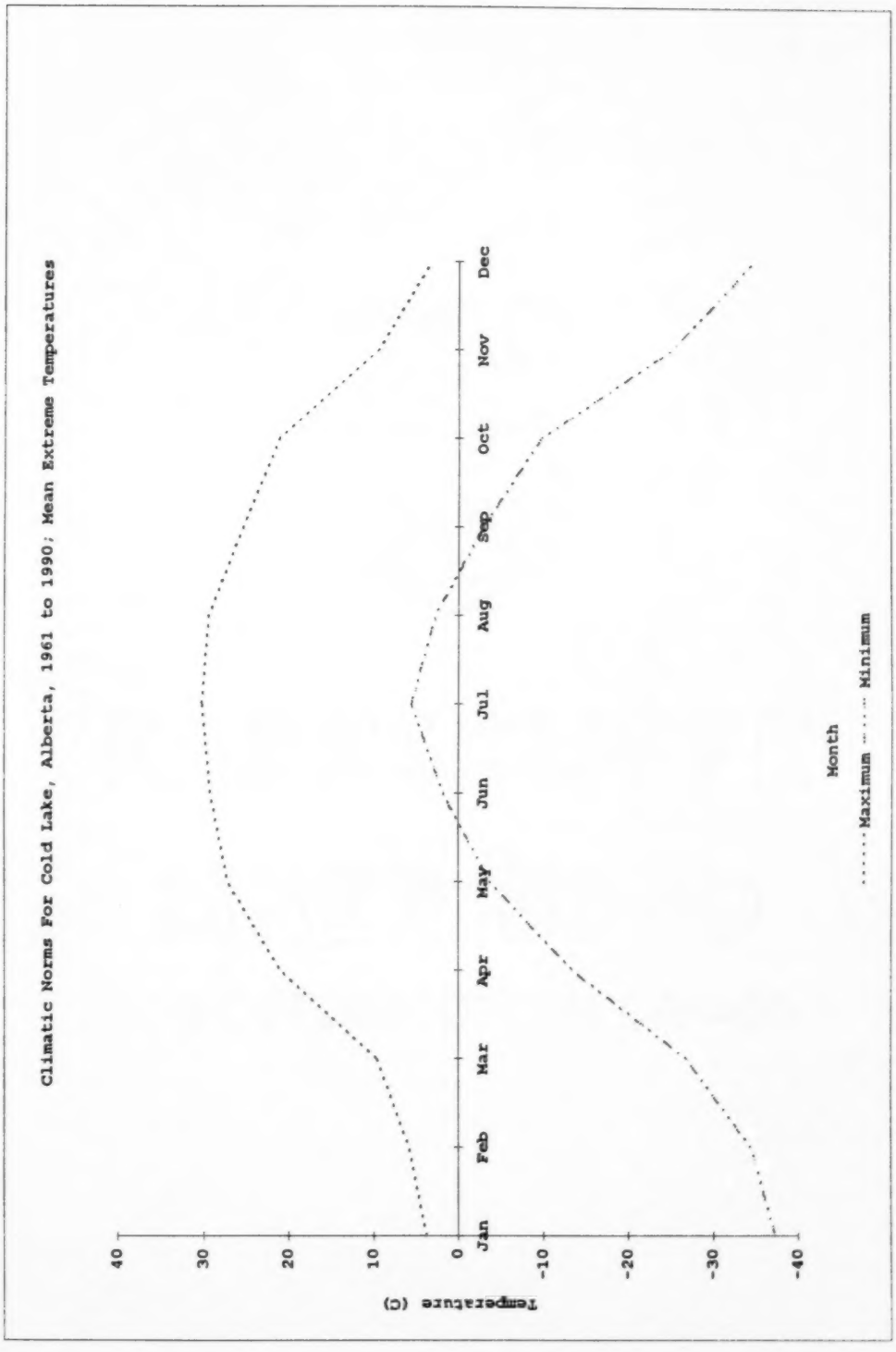

Climatic Summary For Cold Lake, Alberta, 1970 to 1995; Mean, Maximum and Minimum Temperatures
Temperature (C)
40
30
20
10
0
-10
-20
-30
-40
-50
1970
1973
1976
1979
1982
1985
1988
1991
1994
Years (Jan, 1970, to June, 1995)
Mean
Maximum
Minimum

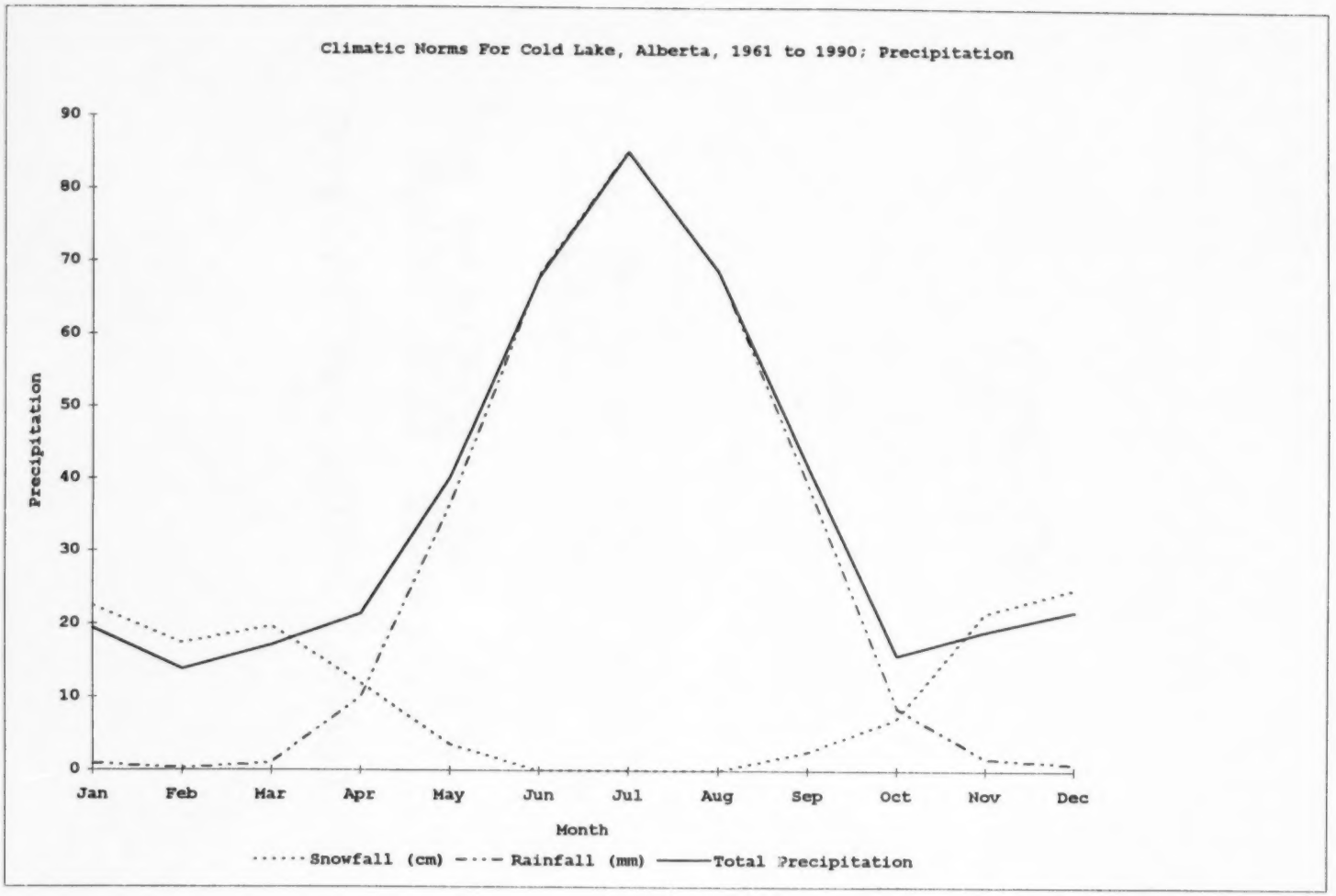

Climatic Summary For Cold Lake, Alberta, 1970 to 1995; Rainfall, Snowfall and Total Precipitation
Precipitation
180
160
140
120
100
80
60
40
20
0
1970
1973
1976
1979
1982
1985
1988
1991
1994
Years (Jan, 1970, to June, 1995)
Snowfall (cm)
Rainfall (mm)
Total (mm)

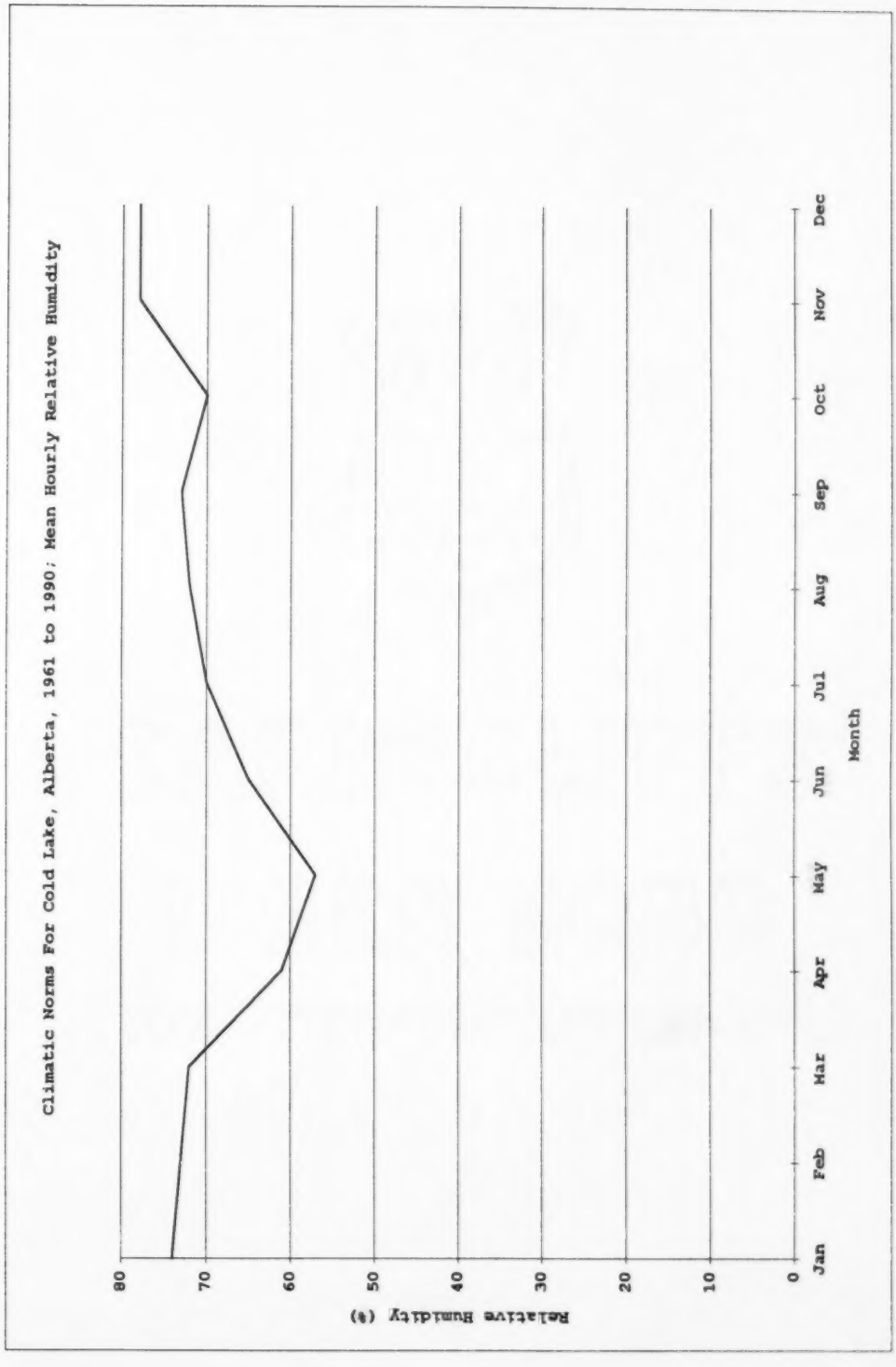
Climatic Norms For Cold Lake, Alberta (1961-1990)

Month	Mean Dly Mx. (C)	Mean Dly Mn. (C)	Daily Mean (C)	Mean Ext. Mx.Tp. (C)	Mean Ext. Mn.Tp. (C)	Mean Snw (cm)	Mean Rn (mm)	Mean Tot. Prcp (mm)	Mean Hrly Rel. Hum.
Jan	-12.4	-22.4	-17.5	3.8	-37.3	22.5	0.9	19.4	74
Feb	-7.4	-19.1	-13.2	5.8	-34.5	17.4	0.3	13.8	73
Mar	-0.8	-12.3	-6.5	9.6	-26.9	19.7	1	17.1	72
Apr	9.5	-2.5	3.6	21	-13.5	11.9	9.9	21.4	61
May	17.2	3.9	10.6	27.2	-3.7	3.6	36.5	40.2	57
Jun	21.1	8.6	14.9	29.2	1.8	0	68.1	67.9	65
Jul	22.9	10.9	17	30.2	5.6	0	85.1	85.1	70
Aug	21.8	9.5	15.7	29.4	2.7	0	68.7	68.7	72
Sep	15.6	3.9	9.8	25.4	-2.9	2.6	39.2	41.8	73
Oct	9.7	-1.1	4.3	21.1	-9.7	7.1	8.6	15.8	70
Nov	-2.6	-11	-6.7	9.4	-25.2	21.7	1.7	19.2	78
Dec	-10.5	-19.7	-15	3.2	-34.8	25	0.9	21.9	78
Annual Mean	7	-4.3	1.4	31.8	-40.2	131.5	320.9	432.2	70


Climatic Norms For Cold Lake, Alberta, 1961 to 1990; Daily Mean, Mean Daily Maximum and Mean Daily Minimum Temperatures
Temperature (C)
25
20
15
10
5
0
-5
-10
-15
-20
-25
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Month
Maximum
Minimum
Mean


Climatic Norms For Cold Lake, Alberta, 1961 to 1990; Mean Extreme Temperatures
Temperature (C)
40
30
20
10
0
-10
-20
-30
-40
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Month
Maximum
Minimum


Climatic Norms For Cold Lake, Alberta, 1961 to 1990; Precipitation
Precipitation
90
80
70
60
50
40
30
20
10
0
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Month
Snowfall (cm)
Rainfall (mm)
Total Precipitation


Climatic Norms For Cold Lake, Alberta, 1961 to 1990; Mean Hourly Relative Humidity
Relative Humidity (%)
80
70
60
50
40
30
20
10
0
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Month